Pharmacological evaluation of disulfiram analogs as antimicrobial agents and their application as inhibitors of fosB-mediated fosfomycin resistance

Alexandria D. Lewis1 · Taylor M. Riedel1 · Meredith B. A. Kesler1 · Melinda E. Varney1 · Timothy E. Long1,2

Received: 30 September 2021 / Revised: 9 November 2021 / Accepted: 29 December 2021 / Published online: 20 January 2022
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Abstract
Disulfide analogs of the alcohol sobriety medication disulfiram (Antabuse®) were evaluated for antimicrobial activity. Structure-activity relationship analyses of MIC data obtained for methicillin-resistant Staphylococcus aureus (MRSA) and other pathogenic organisms revealed correlations between the lipophilicity and bulkiness of the substituents. Analogs conferring optimal anti-MRSA activity contained S-octyl disulfides and either N,N-dimethyl- or N-pyrrolidine dithiocarbamate substituents. Additional testing revealed that both disulfiram and its S-octyl derivative are capable of sensitizing S. aureus to the bactericidal effects of fosfomycin. Mechanistic studies established that the compounds decrease intracellular levels of the fosB cofactor bacillithiol through a thiol-disulfide exchange reaction. The increased fosfomycin susceptibility in S. aureus was thereby attributed to a depleted cellular bacillithiol pool available for inactivation by fosB.

Introduction
Methicillin-resistant Staphylococcus aureus (MRSA) is among the most common pathogens implicated in healthcare-associated infections [1]. Despite efforts to reduce hospital transmission rates, there is a continual need to develop new treatments for combating invasive infections due to vancomycin-susceptible (VSSA) and less frequent vancomycin-intermediate resistant (VISA) and vancomycin-resistant S. aureus (VRSA). Among the approaches to develop new antimicrobial therapies are repurposing and reconfiguring the chemical structure of medications approved for human use [2]. Respective examples include application of the alcohol sobriety aid disulfiram (Antabuse™) [3] and derivatives [4] as antibiotic adjuvants in the treatment of multidrug-resistant S. aureus infections. As a pleiotropic drug with sulfhydryl-modifying and chelating properties, disulfiram has the ability to be repositioned as a treatment for other disease states (e.g. infection, cancer).

Prior antimicrobial evaluation of disulfiram (DSF) and S-alkyl N,N-diethylthiocarbamates (DDTC) derivatives revealed the disulfides to possess narrow spectrum activity against S. aureus and other Gram-positive bacteria [4]. Structure-activity relationship (SAR) analysis on the derivatives established distinct correlations between the S-alkyl chain length and minimum inhibitory concentrations (MICs). Disulfides with linear and branched S-alkyl chains of five carbons and less were weaker inhibitors of MRSA growth than their linear carbon chain counterparts of six to eight carbons. The most active analog in the DSF-derived series was identified as S-octyl disulfide 2d (R, R’ = Et, n = 7) (Fig. 1). In the current study, the effect of N,N-substitution on the antimicrobial activity of the disulfides was investigated. Pharmacological studies on their ability to alter intracellular thiol levels and impair fosfomycin inactivation in S. aureus were also examined.
Materials and methods

Chemicals for the synthesis of compounds 1–16 were acquired from commercial sources and used as received. Products were purified on Biotage® SNAP KP-Sil silica gel columns using an automated Isolera™ chromatography system with elution detection set at λ 254 nm. Mass spectra were recorded on an Agilent 5977B GCMS. 1H and 13C NMRs were recorded on a Bruker AVANCE™ III HD 300 MHz spectrometer and referenced to TMS. Bacillithiol (BSH) trifluoroacetic acid salt and monobromobimane were purchased from Biosynth Carbosynth (United Kingdom) and Cayman Chemical (Ann Arbour, MI), respectively.

Chemistry

Preparation of disulfides 1, 2, and 16

The respective thiols (1.0 mmol) and thiuram disulfides (1.0 mmol) were combined in 5 ml of dry N,N-dimethylformamide (DMF). The reaction mixtures were stirred for 2 h at 60 °C (Fig. 1). The solutions were then cooled to room temperature, diluted with 5 ml of ethyl acetate, washed five times with 5% citric acid, and dried over MgSO4. The filtered organic layers were concentrated in vacuo and the products were purified on silica gel using a 5–50% gradient of ethyl acetate in hexanes. Characterization data of representative compound ethyl dimethylcarbamo(dithioperoxo)thioate (1a): oil (34%, 62 mg); TLC (SiO2) Rf 0.55 (4:1 hexanes:EtOAc); 1H NMR (300 MHz, CDCl3) δ 3.64 (s, 3H), 3.51 (s, 3H), 2.99 – 2.82 (m, 2H), 1.35 (t, J = 7.4 Hz, 3H); 13C NMR (75 MHz, CDCl3) δ 197.5, 47.2, 41.6, 32.2, 13.7; MS (EI, 70 eV): m/z = 181.0 [M]+.

Preparation of disulfides 3–14

Thiols (0.83 mmol) were added dropwise to 15 ml centrifuge tubes containing sulfonyl chloride (60 µl, 0.75 mmol) in 4 ml dry dichloromethane. The solutions were shaken at 1000 rpm for 0.5 h at 5 °C using a Benchmark Scientific MultiTherm Shaker™ as a parallel synthesis reactor to generate the sulfonyl chlorides in situ. Separately, the amines (0.83 mmol) were combined with carbon disulfide (50 µl, 0.83 mmol) and N,N-diisopropylethylamine / Hünig’s base (144 µl, 0.83 mmol) in a microfuge tube containing 1 ml of dry diethyl ether. The amine solutions were vortexed for 5 min at room temperature and added dropwise to the shaking solutions of sulfonyl chlorides at 5 °C. After 1 h, the reactions were quenched with 4 ml of 1 N HCl and vortexed. The top aqueous layers were removed by pipette the organic layers were washed with 4 ml saturated Na2CO3 and dried over MgSO4. The filtered organic layers were concentrated in vacuo and the products were purified on silica gel using 0-10% gradient of ethyl acetate in hexanes. Characterization data of representative compound octyl pyrrolidine-1-carbo(dithioperoxo)thioate (10d): solid (39%, 94 mg), m.p. 31-32 °C; TLC (SiO2) Rf 0.67 (4:1 hexanes:EtOAc); 1H NMR (300 MHz, CDCl3) δ 3.96 (t, J = 6.9 Hz, 2H), 3.75 (t, J = 6.8 Hz, 2H), 2.93 – 2.79 (m, 2H), 2.18 – 2.06 (m, 2H), 2.06 – 1.93 (m, 2H), 1.72 – 1.60 (m, 2H), 1.46 – 1.19 (m, 10H), 0.93 – 0.82 (m, 3H); 13CN M R (75 MHz, CDCl3) δ 192.8, 56.6, 50.5, 39.5, 38.6, 31.4, 29.2, 28.6, 28.2, 26.5, 24.2, 22.5, 14.0; MS (EI, 70 eV): m/z = 291.0 [M]+.

Biology

Antimicrobial testing

Minimum inhibitory concentrations (MICs) were determined by the microdilution assay method in cation-adjusted Mueller-Hinton broth (CAMHB) for bacteria [5] and RPMI-1640 medium supplemented with 2% glucose and buffered with 0.165 M morpholinepropanesulfonic acid (MOPS) to pH 7.0 for Candida albicans [6]. Overnight cultures of bacteria and C. albicans adjusted to an initial inoculum of...
10^5 and 10^3 cfu ml^-1, respectively, were treated with two-fold dilutions of test compounds in 96 well plates. Following 24 h incubation in a water-jacketed incubator, the MICs were recorded as the lowest drug concentration that conferred complete inhibition of visual growth. For evaluation of bactericidal effects, 5 µl from the 24 h CAMHB cultures were inoculated on Mueller-Hinton agar using a multichannel pipette and inspected for growth following overnight incubation.

**Thiol metabolomic studies**

Intracellular thiol levels were measured by HPLC with fluorescent detection (FLD) using the sulfhydryl-labeling reagent monobromobimane [7]. An overnight culture of S. aureus JE2 (1 ml) added to 500 ml of LB broth was grown to an OD_{600} density of 1.45. The culture was then dispensed as 15 ml aliquots into 15 ml tubes containing the respective test compounds from 1 mg ml^-1 stocks. The tubes were incubated for 1 h with shaking (37 °C, 200 rpm), centrifuged (5 K rpm, 5 °C, 5 min), and the broth was decanted. The bacterial pellets were transferred to 2 ml centrifuge tubes with 1.6 ml sterile saline, centrifuged (14 K rpm, 5 °C, 2 min), and the supernatant was removed by pipette. The pellets were then treated with 200 µl of 2 mM monobromobimane in 50% acetonitrile with 0.025 M HEPES (pH 8.0) and incubated with shaking (60 °C, 500 rpm) in the dark. After 15 min, 10 mM methanesulfonic acid (800 µl) was added after cooling, the samples were centrifuged, and the supernatants were transferred to vials for FLD HPLC analysis using the separation methods described in the supplemental section. Cellular thiol levels were calculated from cell pellet dry weights and calibration curves derived from reference standard concentrations of 0.01, 0.05, 0.1, 0.5, 1.0, and 2.0 µM.

**Statistical analyses**

Statistical significance was assessed by analysis of variance (ANOVA) with Tukey’s posttest for multiple comparisons using Prism 9.0.2 (GraphPad Software, Inc) software. P values of <0.05 were deemed significant.

**Results**

**Synthesis of disulfiram analogs**

N,N-Dimethyl- (1) and N,N-diethyl- (2) dithiocarbamates were synthesized from thiram and disulfiram (DSF), respectively, via a thiol-disulfide exchange reaction (Fig. 1) [8]. In a newly developed route, S-alkyl dithiocarbamates 3-14 were prepared in parallel synthesis format from their corresponding N,N-dialkylamines or cyclic amines, carbon disulfide, and sulfonyl chlorides (Fig. 1). The later was generated from sulfuryl chloride and an alkyl thiol then treated in situ with dithiocarbamate ammonium salts prepared from the secondary amines and carbon disulfide in the presence of Hünig’s base.

**Antimicrobial susceptibility testing**

Table 1 presents the antimicrobial susceptibility data for thirty-six dithiocarbamate disulfides 1-9 bound with linear and branched dialkylamino moieties. A correlation between the degree of lipophilicity, as defined by clogP, and antibacterial activity was established for dithiocarbamates 1-7. Analogs with clogP values less than 7 exhibited the greatest anti-MRSA activity with a minimum inhibitory concentration (MIC) range of 0.5–16 µg ml^-1. The length and bulk of the N,N-dialkylamino substituents accordingly had a profound influence on antimicrobial activity as illustrated by the stark difference between the MICs of N-benzyl analogs 8 and 9.

N-Methyl- (1, 9) and N-ethyl- (2) dithiocarbamate-substituted analogs were identified as the most efficacious inhibitors of overall bacterial growth (Table 1). Within each series, the S-octyl disulfides (1d, 2d, 9d) exhibited optimal MICs compared to their shorter chain counterparts against three S. aureus variants (VSSA, VISA, VRSA) and other Gram-positive cocci (i.e., VISE, VRE). Shorter chain derivatives were by contrast more active inhibitors of Gram-positive bacteria growth among dithiocarbamates containing bulkier N,N-dialkylamino residues (3-8).

Further antimicrobial testing revealed Gram-negative bacteria Acinetobacter baumannii and Escherichia coli were non-susceptible at MICs ≥ 8 µg ml^-1 with exception of S-ethyl thiram analog 1a (MICs 4 µg ml^-1). The derivatives were also tested as antifungal agents as prior research showed that DSF exhibits activity against yeast and molds [9]. Shorter chain disulfides (n = 1, 3) were likewise the most efficacious growth inhibitors of C. albicans with the S-ethyl analog 2a demonstrating comparable potency to caspofungin.

In addition, a second series of analogs derived from cyclic amines (10-14) was evaluated against the microbial panel for comparison. It was rationalized that conformational constraints imposed by the rings may confer greater activity due to reduction of rotatable bonds [10]. Accordingly, pyrrolidine- (10), piperidine- (11), and azepane- (12) based dithiocarbamates displayed similar potencies to analogs 1 and 2 against S. aureus irrespective of ring size (Table 2). The S-alkyl chain length within each series had marginal influences on the activity with S-ethyl and S-decyl.
| Compd | Structure | Species<br>\( ^{\text{a}} \) MIC (µg ml\(^{-1}\)) | E. coli | A. baumannii | C. albicans |
|-------|-----------|-------------------------------------------------|--------|-------------|-------------|
| 1a    | Me Me     | 1                                              | 2.30  | 2           | 1           | 1           | 4           | 8           | 4           | 4           | 0.5         |
| 1b    | Me Me     | 3                                              | 3.36  | 2           | 1           | 1           | 4           | 4           | 16          | 8           | 0.5         |
| 1c    | Me Me     | 5                                              | 4.42  | 1           | 1           | 1           | 2           | 4           | >32         | 32          | 1           |
| 1d    | Me Me     | 7                                              | 5.48  | 0.5         | 0.5         | 0.5         | 1           | 2           | >32         | 32          | 1           |
| thiram| Me Me     | —                                              | 1.76  | 2           | 2           | 2           | 4           | 8           | >16         | 8           | 1           |
| 2a    | Et Et     | 1                                              | 3.36  | 2           | 1           | 2           | 4           | 8           | 32          | 8           | ≤0.25       |
| 2b    | Et Et     | 3                                              | 4.42  | 2           | 1           | 2           | 4           | 8           | >32         | >32         | 0.5         |
| 2c    | Et Et     | 5                                              | 5.48  | 1           | 1           | 1           | 2           | 4           | >32         | >32         | 0.5         |
| 2d    | Et Et     | 7                                              | 6.53  | 1           | 1           | 1           | 4           | >32         | >32         | 1           |
| disulfiram| Et Et  | —                                              | 3.88  | 16          | 4           | 16          | 4           | 16          | >16         | 16          | 2           |
| 3a    | n-Pr n-Pr | 1                                              | 4.42  | 16          | 16          | 16          | 8           | 16          | >32         | 32          | 2           |
| 3b    | n-Pr n-Pr | 3                                              | 5.48  | 16          | 16          | 16          | 8           | 16          | >32         | >32         | 4           |
| 3c    | n-Pr n-Pr | 5                                              | 6.53  | 4           | 4           | 4           | 2           | 32          | >32         | >32         | 8           |
| 3d    | n-Pr n-Pr | 7                                              | 7.59  | >16         | >16         | >16         | 8           | >16         | >16         | >16         | 16          |
| 4a    | n-Bu n-Bu | 1                                              | 5.45  | 16          | 4           | 8           | 4           | 16          | >32         | >32         | 1           |
| 4b    | n-Bu n-Bu | 3                                              | 6.53  | 16          | 4           | 8           | 2           | 32          | >32         | >32         | 2           |
| 4c    | n-Bu n-Bu | 5                                              | 7.59  | >16         | >16         | >16         | 16          | >16         | >16         | >16         | 16          |
| 4d    | n-Bu n-Bu | 7                                              | 8.65  | >16         | >16         | >16         | >16         | >16         | >16         | >16         | 8           |
| 5a    | i-Bu i-Bu | 1                                              | 5.22  | 16          | 8           | 8           | 4           | 32          | >32         | >32         | 1           |
| 5b    | i-Bu i-Bu | 3                                              | 6.27  | 16          | 8           | 8           | 2           | 32          | >32         | >32         | 2           |
| 5c    | i-Bu i-Bu | 5                                              | 7.33  | >32         | 16          | 16          | 32          | >32         | >32         | >32         | 2           |
| 5d    | i-Bu i-Bu | 7                                              | 8.39  | >32         | 32          | >32         | >16         | >32         | >32         | >32         | 32          |
| 6a    | n-Hex n-Hex | 1                                             | 7.59  | 16          | 16          | 16          | 4           | >16         | >16         | >16         | 1           |
| 6b    | n-Hex n-Hex | 3                                             | 8.65  | >16         | >16         | >16         | 16          | >16         | >16         | >16         | 4           |
| 6c    | n-Hex n-Hex | 5                                             | 9.71  | >16         | >16         | >16         | >16         | >16         | >16         | >16         | 16          |
| 6d    | n-Hex n-Hex | 7                                             | 10.71 | >16         | >16         | >16         | >16         | >16         | >16         | >16         | >16         |
| 7a    | n-Oct n- Oct | 1                                          | 9.71  | >16         | >16         | >16         | >16         | >16         | >16         | >16         | 4           |
| 7b    | n-Oct n- Oct | 3                                          | 10.77 | >16         | >16         | >16         | >16         | >16         | >16         | >16         | 16          |
| 7c    | n-Oct n- Oct | 5                                          | 11.82 | >16         | >16         | >16         | >16         | >16         | >16         | >16         | 16          |
| 7e    | n-Oct n- Oct | 7                                          | 12.88 | >16         | >16         | >16         | >16         | >16         | >16         | >16         | >16         |
| 8a    | Bn Bn     | 1                                              | 6.30  | 4           | 1           | 2           | 2           | 8           | >32         | >32         | 2           |
| 8b    | Bn Bn     | 3                                              | 7.36  | 16          | 4           | 4           | 4           | 2           | 32          | >32         | 16          |
| 8c    | Bn Bn     | 5                                              | 8.42  | >16         | >16         | >16         | 16          | >16         | >16         | >16         | >16         |
| 8d    | Bn Bn     | 7                                              | 9.48  | >16         | >16         | >16         | >16         | >16         | >16         | >16         | >16         |
| 9a    | Bn Me     | 1                                              | 4.53  | 4           | 4           | 4           | 4           | 8           | 32          | 8           | 2           |
| 9b    | Bn Me     | 3                                              | 5.59  | 2           | 2           | 4           | 2           | 8           | >32         | >32         | 4           |
| 9c    | Bn Me     | 5                                              | 6.65  | 0.5         | 0.5         | 0.5         | 1           | 4           | >32         | >32         | 32          |
| 9d    | Bn Me     | 7                                              | 7.71  | 8           | 4           | 32          | 2           | 32          | >32         | >32         | >32         |
| vancomycin | —        | —                                              | —     | —           | —           | —           | —           | —           | —           | —           | —           |
| colistin  | —        | —                                              | —     | —           | —           | —           | —           | —           | —           | 0.5         | —           |
| caspofungin | —        | —                                              | —     | —           | —           | —           | —           | —           | —           | —           | ≤0.25       |

\(^{\text{a}}\)Calculated partition coefficient.

\(^{\text{b}}\)Vancomycin-susceptible, methicillin-resistant *Staphylococcus aureus* N315 (VSSA/MRSA); vancomycin-intermediate resistant *S. aureus* AR-19 (VISA); vancomycin-resistant *S. aureus* HIP14300 (VRSA-4); vancomycin-intermediate *Staphylococcus epidermidis* NR6 (VISE); vancomycin-resistant *Enterococcus faecalis* TX0104 (VRE); *Escherichia coli* K-12, Strain DC10B; *Acinetobacter baumannii* WC-136; *Candida albicans* P78048.
derivatives showing increased MICs. Conversely, the effect of the S-alkyl chain length was more evident for the morpholine- (13) and thiomorpholine- (14) based analogs. Those bound with shorter chain S-ethyl and S-butyl groups were comparably weaker inhibitors of Gram-positive bacteria growth than their longer chain counterparts. An opposite trend was observed for A. baumannii, E. coli, and C. albicans, which again were the most susceptible to short chain S-ethyl disulfide analogs.

Cytotoxicity studies

Select disulfides and DSF were evaluated for cytotoxicity in human embryonic kidney cells (HEK-293). Negligible differences in cell viability via the MTT assay were observed for compounds 1d, 12c, and DSF compared to vehicle control (Fig. 1S). By comparison with DSF and vehicle, analog 10d exhibited moderately greater suppression of HEK-293 growth after 24 h that was statistically significant at concentrations 0.25–2 μg ml⁻¹.

Pharmacological studies

Low molecular weight thiols are used by cells to maintain redox homeostasis and protect against oxidative stress [11]. Bacillithiol (BSH) is the primarily thiol to serve this function in S. aureus and the BSH system is considered a potential target for therapeutic development [12]. Prior work has shown that S. aureus devoid of BSH had decreased survival in blood [13] and increased sensitivity to the bactericidal antibiotic fosfomycin (FOS) [14]. Mechanistic studies on FOS resistance in S. aureus have established that strains harboring fosB utilize BSH to inactivate FOS in an epoxide-ring opening reaction mediated by the S-transferase gene product (Fig. 2a) [12].

In a previous report [4], DSF was found to lower the MIC of FOS against wildtype (WT) JE2, a BSH- and fosB-producing MRSA strain. In combination with FOS, a significant decrease in MIC was similarly observed with derivative 10d (Table 3). To probe if either BSH or fosB is a target of 10d, isogenic JE2 transposon mutants ∆bshA.
Fig. 2  
(a) Mechanisms of fosfomycin inactivation by bacillithiol [12] and (b) thiol-disulfide exchange reactions for disulfiram and analog 10d

Table 3 MIC comparison of disulfiram (DSF), vancomycin (VAN), fosfomycin (FOS), and analog 10d for JE2 and isogenic BSH and fosB transposon mutants

| Strain         | MIC (µg ml\(^{-1}\)) | 10d | DSF | FOS | VAN | 10d\(^a\) | DSF\(^a\) |
|----------------|-----------------------|-----|-----|-----|-----|---------|---------|
| JE2 WT         | 1                     | 16  | 16 – 32 | 1   | 0.25 | 4       |
| JE2 ΔbshA      | 0.5                   | 8 – 16 | 8   | 1   | <0.25 | <0.25  |
| JE2 ΔfosB      | 1                     | 16  | 8   | 1   | <0.25 | <0.25  |

\(^a\)MIC in CAMHB supplemented with 8 µg ml\(^{-1}\) FOS.

(NE1728) and ΔfosB (NE1479) were evaluated [15]. Susceptibility testing revealed increased sensitivity for the BSH-null mutant and no change in MIC for the fosB-null mutant in comparison with WT JE2. Moreover, the combination of 10d and DSF with FOS conferred increased bactericidal effects against the three test strains in an apparent BSH-dependent manner. Figure 3 shows that JE2 mutant ΔbshA lacking BSH was significantly more susceptible compared to parent JE2 and ΔfosB strains. The lethal effects conferred in the ΔbshA strain suggested that BSH is a cellular target of the compounds.

With these findings, evidence that 10d and DSF alter the levels of BSH and other cellular thiols was sought. Thiol metabolomics was therein performed on WT JE2 treated with 10d, DSF, FOS, and pyrrolidine dithiocarbamate (PDTC), the byproduct of a thiol-disulfide 10d exchange reaction (Fig. 2b). HPLC analyses using the thiol-reactive fluorescent probe monobromobimane [12] revealed significant decreases in the cytosolic thiol levels of BSH and CYS, but not coenzyme A (CoA), for cultures treated with 10d and DSF (Fig. 4). Conversely, increases in BSH and
CoA levels were observed with PDTC, which may be due to the antioxidant properties of the dithiocarbamate [16]. An increase in CoA levels in FOS-treated cultures was similarly observed; however, no significant changes in cellular BSH and CYS were detected following 1 h treatment.

Further corroboration that the compounds modify *S. aureus* BSH was accomplished using a DSF analog with a fluorescent tracer bound to the S-alkyl chain. The probe was synthesized from dansyl chloride and cysteamine disulfide followed by DTT reduction [17] and a thiol-disulfide exchange reaction of intermediate 15 with dipyrroli-dithiuram disulfide to yield the fluorescent analog 16 (Fig. 5). For the experiment, a commercial BSH standard was used to optimize a HPLC method to distinguish it from CYS. Figure 5 shows the HPLC results for the cellular extract of *S. aureus* JE2 treated with 10 µg ml⁻¹ of disulfide 16 for 1 h in LB broth. Peaks corresponding to labeled BSH and CYS were detected confirming *S. aureus* uptake of the DSF analog and its reaction with intracellular BSH.

**Discussion**

This investigation concluded an in-depth SAR study on the microbiological activity of dithiocarbamate disulfides based on the DSF thiuram pharmacophore. Derivatives with optimal anti-MRSA activity possessed S-octyl chains and either *N,N*-dimethyl- or *N*-pyrrolidine-bound dithiocarbamates. Further evaluation of pyrrolidine analog 10d against additional MRSA isolates (*n* = 10) gave a MIC₉₀ of 1 µg ml⁻¹ with a MIC range of 0.5–1 µg ml⁻¹. *A. baumannii*, *E. coli*, and *C. albicans* on the other hand were most vulnerable to short chain S-ethyl analogs. A cellular feature common to these microbes, but not MRSA, is their primary use of glutathione (GSH) in the maintenance of redox homeostasis and ROS detoxification [11]. Prior studies have shown that DSF forms a mix disulfide with GSH (i.e., GS-DDTC) that is further metabolized by GSH to GS-SG [18, 19]. In *A. baumannii*, *E. coli*, and *C. albicans*, the MIC differences between DSF and the S-ethyl analogs may be attributed to slower regeneration of GSH from the GS-SEt exchange product compared to GS-DDTC.

In Gram-positive bacteria, alternative thiols such as BSH are used to preserve redox homeostasis. Figure 6 depicts the proposed mechanisms of BSH-disulfide exchange for DSF and analog 10d in *S. aureus*. Reactions with DSF are believed to form an unstable BS-DDTC adduct that is vulnerable to BSH cleavage. The resulting disulfide BS-SB may then be reduced by BSH reductase YpdA to recover BSH [20]. By comparison, the exchange reaction between 10d and BSH is believed to yield a more stable disulfide, (i.e., BS-SO₅). The ability to suppress BSH levels for a longer duration than DSF may partially account for the greater inhibition of *S. aureus* growth observed with analog 10d.

Although disruption of the BSH system is believed to play a key role in the growth inhibition, the distinct SAR of the DSF analogs suggests involvement of other mechanisms. Previously studied diallyl thiosulfinate (allicin) is an electrophilic disulfide that also undergoes thiol-disulfide exchange reaction with BSH and was found to modify fifty-seven proteins in *S. aureus* including those involved in redox processes and metabolism [21]. Thus, oxidative stress induction and metabolic perturbations are believed to be contributing factors. It was further reported that reversal of the protein S-thioallylation is mediated by the Brx/BSH/YpdA system wherein Brx facilitates translocation of the S-allyl adduct to BSH and YpdA regenerates BSH from the resulting BS-S-allyl product [21]. This same pathway would presumably be used to detoxify proteins modified by the DSF analogs at CYS residue sites. Growth inhibition may therefore also correlate to the efficiency of Brx/BSH/YpdA to remove the S-alkyl adducts from proteins in *S. aureus*.

In addition, the reaction of intracellular thiols with DSF and the analogs results in the generation of dithiocarbamate anions (e.g., DDTC, PDTC) that have potential...
growth-inhibiting, metal-chelating properties (Fig. 6). Both DDTC and PDTC have been shown to possess metal-dependent antibacterial activity against Gram-positive species [4, 22, 23]. The ability of dithiocarbamate anions to inhibit metalloenzymes or extract their metal cofactors may therefore further contribute to the antimicrobial effects conferred by DSF and the analogs.

A final objective of the study was to examine the relationship between BSH inhibition and FOS efficacy against S. aureus. Analog 10d and DSF were found to increase MRSA susceptibility to FOS when combined with sub-MIC amounts of either disulfide effect that was attributed to reductions in cellular BSH levels. The discovery that DSF may restore FOS susceptibility could have a therapeutic utility and as a medication approved for human use, future investigations will evaluate its potential as an antibiotic adjuvant for the treatment of FOS-resistant infections.

Acknowledgements This work was supported by the National Institute of Allergy and Infectious Diseases, National Institutes of Health under award number AI151970. Test organisms (Table 1S) were acquired from the American Type Culture Collection, FDA-CDC Antimicrobial Resistance Isolate Bank, and the Network on Antimicrobial Resistance in Staphylococcus aureus (NARSA) for distribution by BEI Resources, NIAID, and NIH.

Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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