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A selective membrane-targeting repurposed antibiotic with activity against persistent methicillin-resistant Staphylococcus aureus


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Treatment of Staphylococcus aureus infections is complicated by the development of antibiotic tolerance, a consequence of the ability of S. aureus to enter into a nongrowing, dormant state in which the organisms are referred to as persisters. We report that the clinically approved anthelmintic agent bithionol kills methicillin-resistant S. aureus (MRSA) persister cells, which correlates with its ability to disrupt the integrity of Gram-positive bacterial membranes. Critically, bithionol exhibits significant selectivity for bacterial compared with mammalian cell membranes. All-atom molecular dynamics (MD) simulations demonstrate that the selectivity of bithionol for bacterial membranes correlates with its ability to penetrate and embed in bacterial-mimic lipid bilayers, but not in cholesterol-rich mammalian-mimic lipid bilayers. In addition to causing rapid membrane permeabilization, the insertion of bithionol increases membrane fluidity. By using bithionol and nTzDpa (another membrane-active antimicrobial agent), as well as analogs of these compounds, we show that the activity of membrane-active compounds against MRSA persisters positively correlates with their ability to increase membrane fluidity, thereby establishing an accurate biophysical indicator for estimating antipersister potency. Finally, we demonstrate that, in combination with gentamicin, bithionol effectively reduces bacterial burdens in a mouse model of chronic deep-seated MRSA infection. This work highlights the potential repurposing of bithionol as an antipersister therapeutic agent.

MRSA | bacterial persister | drug repurposing | membrane-active antimicrobials | membrane selectivity

Staphylococcus aureus is a Gram-positive opportunistic human pathogen carried by approximately one third of the human population. Despite antibiotic availability, S. aureus infections are often hard to cure and remain one of the major causes of death (1), in part because of the ability of S. aureus to enter into a nongrowing antibiotic-tolerant state, in which the organisms are referred to as persisters (2). Persisters exhibit significantly reduced biosynthetic processes, which are the major targets for most antibiotics (2). Persisters also exist in a metabolically low-energy state (3) that prevents the energy-dependent uptake of antibiotics such as aminoglycosides (4). S. aureus persisters are present in high numbers in stationary-phase suspension cultures and biofilms (4–6) and are responsible for chronic and relapsing infections such as endocarditis, osteomyelitis, and prosthetic implant infections (3).

Bacterial membranes are attractive antipersister targets because they can be disrupted independently of growth. Although membrane-active agents are typically toxic to mammals because of low membrane selectivity (7), the clinical success of daptomycin has sparked new interest in membrane-active antimicrobial therapeutic agents (7). The lipopeptide tail of daptomycin, a natural cyclic lipopeptide synthesized by Streptomyces roseosporus, inserts into Gram-positive bacterial membranes, forming oligomeric pores, causing membrane depolarization, potassium ion efflux, and rapid cell death (8). Despite strong antimicrobial potency against growing bacterial cells, daptomycin has not been reported to be effective against persisters (9, 10).

Recently, our laboratory described the identification of membrane-active synthetic retinoids that are efficacious in killing MRSA

Significance

There is a critical lack of therapeutic agents to treat infections caused by nongrowing persister forms of methicillin-resistant Staphylococcus aureus (MRSA). Although membrane-disrupting agents can kill persister cells, their therapeutic potential has been mostly overlooked because of low selectivity for bacterial versus mammalian membranes. We report that the clinically approved anthelmintic drug bithionol kills MRSA persisters by disrupting membrane lipid bilayers at concentrations that exhibit low levels of toxicity to mammalian cells. The selectivity of bithionol results from the presence of cholesterol in mammalian but not in bacterial membranes. We also show that the antipersister potency of membrane-active antimicrobial agents correlates with their ability to increase membrane fluidity. Our results significantly enhance our understanding of bacterial membrane disruption and membrane selectivity.


Conflict of interest statement: F.M.A. and E.M. have financial interests in Germa Biosciences and Octagon Therapeutics, companies that are engaged in developing antimicrobial compounds. E.M.’s and F.M.A.’s interests were reviewed and are managed by Rhode Island Hospital (E.M.) and Massachusetts General Hospital and Partners HealthCare (F.M.A.) in accordance with their conflict of interest policies. The remaining authors declare no competing financial interests.

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persisters (6). They are also relatively nontoxic because they exhibit a significant amount of selectivity for Gram-positive bacterial membranes compared with mammalian membranes (6). Like daptomycin, the antimicrobial retinoids also appear to kill nongrowing MRSA persisters cells (6). In addition to the synthetic retinoids, we have described 2 additional membrane-active compounds with anti-MRSA activity: NH125, a histidine kinase inhibitor (11), and nTZDpa, a nonthiazolidinedione peroxisome proliferator-activated receptor gamma partial agonist (12). Although NH125 and nTZDpa have excellent anti-MRSA persister activity, they both cause substantial hemolysis of red blood cells at high concentrations (>32 μg/mL) (12, 13), which is a drawback for further development as anti-MRSA therapeutic agents.

The membrane-active retinoids, NH125, and nTZDpa were identified by screening ~82,000 synthetic compounds by using a high-throughput Caenorhabditis elegans–MRSA infection screening assay for compounds that block the ability of MRSA to kill the nematodes (6, 11, 12). We subsequently screened 185 “hit” compounds for the additional ability to permeabilize MRSA persisters by using a SYTOX Green membrane permeability assay (11). Another putative membrane-active antimicrobial identified in this screen was the clinically approved antimalarial drug bithionol, which is chosen for additional studies because of its well-established pharmacokinetic, toxicity, and safety profiles (14). In this paper, we describe the therapeutic potential of bithionol as an anti-MRSA persister antibiotic agent and provide a putative mode of action that explains the ability of bithionol to selectively disrupt bacterial compared with mammalian membrane lipid bilayers. Moreover, by studying a panel of bithionol and nTZDpa analogs, we found a strong correlation between the potency of anti-MRSA persister activity and the ability to increase membrane fluidity.

Results

Bithionol Shows Bactericidal Activity Against both Antibiotic-Resistant S. aureus and S. aureus Persister Cells. Bithionol (Fig. 1A) was previously described as an antimicrobial agent with a minimal inhibitory concentration (MIC) of ~8 to 15 μg/mL against Gram-positive bacteria, including S. aureus (15). We confirmed that bithionol exhibits antibacterial activity against a variety of Gram-positive pathogens, including vancomycin-resistant S. aureus (VRSA) and vancomycin- and daptomycin-resistant enterococcal strains. In our hands, however, bithionol was more potent than previously reported, with MICs of 0.5 to 2 μg/mL, comparable to daptomycin (SI Appendix, Table S1). We also confirmed that bithionol exhibits relatively weak antimicrobial activity against Gram-negative pathogens (MICs of 16 to >64 μg/mL; SI Appendix, Table S1).

In contrast to a previous study that described bithionol as bacteriostatic (16), we found that bithionol eradicated ~10^5 CFU/mL exponential-phase S. aureus strain MW2 within 3 h at 10 μg/mL (10× MIC; SI Appendix, Fig. S1A). The rate of killing was comparable to daptomycin (at 10× MIC) and significantly faster than the killing kinetics of vancomycin (at 10× MIC; SI Appendix, Fig. S1A). Consistent with its bactericidal activity, 10 μg/mL bithionol caused a time-dependent decrease in optical density of S. aureus cells comparable to the antiseptic detergent benzylidimethylhexadecylammonium chloride (16-BAC; SI Appendix, Fig. S1B), indicating that bithionol has bactericidal activity. Indeed, transmission electron micrographs (TEMs) showed that 10× MIC bithionol disrupts MRSA membranes, causing the intracellular formation of mesosome-like structures, abnormal cell divisions, and cell lysis (Fig. 1B), which we previously observed when S. aureus cells were treated with membrane-active antimicrobials (12).

We also found that bithionol killed stationary-phase MRSA MW2 planktonic and biofilm persisters in a dose-dependent manner and completely eradicated them at 32 μg/mL (32× MIC) within 2 h and 24 h, respectively (Fig. 1C and D). In contrast, MW2 persisters displayed a high level of tolerance to 100× MIC of several conventional antibiotics, including daptomycin and linezolid, which were tested for activity against planktonic persisters. Similarly, bithionol killed vancomycin-resistant S. aureus (VRSA) strain VRSA1 persisters, whereas linezolid and daptomycin exhibited no and nominal activity, respectively (Fig. 1E).

Bithionol Interacts with and Disrupts the Bacterial Mimetic Lipid Bilayer. We performed all-atom molecular dynamics (MD) simulations of bithionol interacting with simulated bacterial membranes to elucidate a potential mechanism of action. The topology and parameters of bithionol for the GROMOS54a7 force field (17) were generated by automated Topology Builder (18). We used the previously established model of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC)/1,2-dioleoyl-sn-glycero-3-phospho(1’-rac-glycerol) (DOPG) at a 7:3 ratio (19) to simulate negatively charged S. aureus membranes (SI Appendix, Materials and Methods). The MD modeling showed that bithionol is initially recruited to the membrane surface by the binding of polar hydroxyl and interactions between nonpolar benzene rings and hydrophobic lipid tails (Movie S1). After penetration, bithionol embeds in the outer leaflet of the lipid bilayer (Fig. 2A and Movie S1). The insertion of
even a single bithionol molecule causes a substantial local increase in lipid bilayer disorder \((SI\text{ Appendix, Fig. S2A and C)}\). These MD simulations demonstrate that the polarity of branch groups and the hydrophobicity of core rings play important roles in membrane attachment and penetration.

We further investigated the effects of bithionol on lipid bilayers by using biomembrane-mimicking giant unilamellar vesicles (GUVs) consisting of a single lipid bilayer with a diameter of 10 to 100 \( \mu \)m. GUVs were constructed by using DOPC/DOPG lipids at a 7:3 ratio as in the MD simulations. Lipid aggregates formed on the surfaces of the GUVs exposed to 1 \( \mu \)g/mL bithionol, and, at 10 \( \mu \)g/mL, the GUVs burst (Fig. 2C and Movies S3–S5), indicating that bithionol interacts with and disrupts a bacterial mimetic lipid bilayer.

**Bithionol Induces Rapid Membrane Permeabilization and an Increase in Membrane Fluidity.** By using a SYTOX Green permeability assay, we found that, in contrast to daptomycin, bithionol induced dose-dependent membrane permeability in both exponential-phase MRSA MW2 cells and stationary-phase MRSA MW2 persisters (Fig. 2D and SI Appendix, Fig. S1C). The fluorescence intensity peaked at 4 \( \mu \)g/mL and then decreased at higher concentrations of bithionol (Fig. 2D), most likely as a consequence of nucleic acids released form lysed cells (20), consistent with the observation that 10 \( \mu \)g/mL bithionol lysed MRSA persisters (Fig. 1B). The dose-dependent effects of bithionol on membrane permeability correlated with its dose-dependent killing kinetics (Figs. 1C and 2D and SI Appendix, Fig. S1C), suggesting that the bactericidal activity of bithionol results from its membrane-disrupting activity.

Insertion of particular membrane disrupting compounds into lipid bilayers is known to cause dramatic changes in membrane fluidity that disrupts the normal liquid-crystalline phase of the membrane (21–23). This results in passive permeabilization, loss of membrane protein functions, leakage of cellular components, and bacterial death (24). We therefore tested whether bithionol alters MRSA MW2 membrane fluidity by utilizing the membrane fluidity-sensitive dye Laurdan (25), which exhibits a fluorescence emission wavelength shift dependent on the amount of adjacent water molecules (25). Because water molecule penetration into lipid bilayers is in turn determined by lipid packing density and lipid bilayer fluidity, bacterial membrane fluidity can be quantified by using the Laurdan generalized polarization (GP) metric: \( \text{GP} = \frac{(I_{440} - I_{490})}{(I_{440} + I_{490})} \), ranging from -1 (most fluid and disordered) to +1 (most rigid and ordered) (25). As shown in Fig. 2E, bithionol at concentrations greater than 8 \( \mu \)g/mL induced a significant decrease in Laurdan GP in S. aureus MW2, similar to 50 mM benzyl alcohol (BA), a known membrane fluidizer. These data are consistent with our observation that bithionol concentrations greater than 16 \( \mu \)g/mL exhibited significant killing activity against MRSA MW2 persisters (Fig. 1C).

**Bithionol Does Not Penetrate Mammalian Membranes.** We used MD simulations to determine whether bithionol specifically penetrates bacterial compared with mammalian membranes. Mammalian membranes are comprised of phospholipids, sphingolipids, and cholesterol (26), but can be modeled as a simplified bilayer composed of the zwiterionic lipid 1,2-palmitoyl-oleoyl-sn-glycero-3-phosphocholine (POPC) mixed with cholesterol ranging from 20 to 50 mol% (27, 28). At a POPC/cholesterol ratio of 7:3, MD simulations using the GROMOS54a7 force field showed that bithionol fails to penetrate the simulated mammalian bilayer (Fig. 2A and B, SI Appendix, Fig. S2B, and Movie S2). Moreover, the energy barrier and transfer energy for bithionol increases with increasing percentages of cholesterol (SI Appendix, Fig. S3A). These results are consistent with our observation that the presence of cholesterol results in a more ordered alignment of the membrane lipids (SI Appendix, Fig. S3D), as well as with configurational and thermodynamic analyses (29, 30), which demonstrate that cholesterol condenses the hydrophobic region of the membrane (SI Appendix, Fig. S3B) and decreases membrane fluidity (SI Appendix, Fig. S3C). An independent MD simulation using the CHARMM force field (31) also showed that bithionol preferentially penetrates bacterial-mimetic compared with mammalian-mimetic lipid bilayers (SI Appendix, Fig. S4).

Consistent with the MD simulations, bithionol did not cause observable effects on GUVs formed with 7POPC/3cholesterol (Fig. 2C and Movies S6–S8). Moreover, bithionol exhibited relatively little hemolytic activity against human erythrocytes, with an HCCP >64 \( \mu \)g/mL \((SI\text{ Appendix, Fig. S5A)}) and did not induce SYTOX Green membrane permeabilization of HKC-8 human
renal proximal cells up to 64 μg/mL (Fig. 2D and SI Appendix, Fig. S5 B and C).

**Structure–Activity Relationships.** The MD simulations described here earlier predicted that 2 key elements for the membrane activity of bithionol are the initial binding to the membrane surface using phenolic hydroxyl groups and membrane perturbation induced by chlorinated benzene (Fig. 24 and Movie S1). To further test this proposed mechanism and to obtain additional insights into the effects of functional groups on the potency of bithionol, we conducted structure–activity relationship (SAR) studies by using a commercially available bithionol analog, Bitin-S, as well as with 7 newly synthesized analogs (Table 1 and SI Appendix, Figs. S6 and S7). The effect of binding affinity on antimicrobial and membrane activity was tested by using Bitin-S, a sulfide derivative of bithionol (32), and the bithionol methoxy analog BT-OMe (Table 1). The polar sulfanyl group of Bitin-S provides additional hydrophilic interactions with lipid heads. Bitin-S exhibited decreased antimicrobial activity (MIC 8 μg/mL) and reduced membrane activity (Table 1 and SI Appendix, Fig. S6). Reducing polarity by substituting methoxy groups for the 2 hydroxyl groups (BT-OMe; Table 1 and SI Appendix, Fig. S6) resulted in complete nullification of both antimicrobial and membrane activity (Table 1 and SI Appendix, Fig. S6), indicating that the phenolic hydroxyl groups are critical for antimicrobial activity.

To test whether the size and polarization of the inserted molecular nucleation can affect the extent of membrane perturbation (12), we substituted alternative halogenes for chlorine. Replacement of chlorine with fluorine resulted in reduced membrane and antimicrobial activities as seen with compounds BT-OF, BT-pF, and BT-opF, whereas substitution with bromine showed similar membrane and antimicrobial activities as bithionol as demonstrated by BT-oBr, BT-pBr, and BT-opBr (Table 1 and SI Appendix, Fig. S7). It is likely that the polarity of the C-F bond in the fluoro derivatives may increase the hydrophilicity of the aryl rings, thus decreasing membrane permeability. The bromine derivatives showed increased energy barrier values of 2 to 3 kJ/mol (SI Appendix, Table S2), indicating that the larger bromine atoms may cause more membrane perturbation than the smaller chlorine and fluorine atoms, although the bromines may be slightly disadvantageous for initial binding and penetration.

### Table 1. Structure–activity relationships for antibiotic activity and membrane activity

<table>
<thead>
<tr>
<th>Compd</th>
<th>R</th>
<th>R'</th>
<th>MIC</th>
<th>PKC</th>
<th>MP</th>
<th>MFI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bithionol</td>
<td>S</td>
<td>OH</td>
<td>Cl</td>
<td>Cl</td>
<td>1</td>
<td>32</td>
</tr>
<tr>
<td>Bitin-S</td>
<td>SO</td>
<td>OH</td>
<td>Cl</td>
<td>Cl</td>
<td>8</td>
<td>&gt;64</td>
</tr>
<tr>
<td>BT-OMe</td>
<td>OMe</td>
<td>Cl</td>
<td>Cl</td>
<td>&gt;64</td>
<td>&gt;64</td>
<td>No</td>
</tr>
<tr>
<td>BT-OF</td>
<td>S</td>
<td>OH</td>
<td>F</td>
<td>Cl</td>
<td>2</td>
<td>&gt;64</td>
</tr>
<tr>
<td>BT-oBr</td>
<td>S</td>
<td>OH</td>
<td>Br</td>
<td>Cl</td>
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<tr>
<td>BT-pF</td>
<td>S</td>
<td>OH</td>
<td>Cl</td>
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<tr>
<td>BT-pBr</td>
<td>S</td>
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<tr>
<td>BT-opF</td>
<td>S</td>
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<td>BT-opBr</td>
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<td>Br</td>
<td>2</td>
<td>&gt;64</td>
</tr>
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</table>
| MIC, minimum inhibitory concentration (in micrograms per milliliter); PKC, persister killing concentration (in micrograms per milliliter) required to kill 5 x 10^7 CFU/mL MRSA persister cells below the limit of detection; MP, membrane permeabilization (determined based on SYTOX Green fluorescence intensity); MFI, membrane fluidity increase (determined based on Laurdan GP measurement).

**Bithionol in Combination with Gentamicin Shows Efficacy in a Mouse Deep-Seated MRSA Infection Model.** We previously showed that the aminoglycoside antibiotic gentamicin, which is used to treat severe chronic MRSA infections despite nephrotoxicity issues (33), exhibits significant synergism with synthetic retinoids (6). Although we found that relatively low concentrations of bithionol (8 μg/mL) do not lead to a significant decrease in the viability of MRSA persisters (Fig. 1 C and D), 8 μg/mL bithionol combined with as little as 2 μg/mL gentamicin completely eradicated MRSA MW2 stationary-phase persister cells (Fig. 3D). Similarly, 8 μg/mL bithionol + 16 μg/mL gentamicin eradicated biofilm persisters (Fig. 3B).

We further tested the efficacy of bithionol in combination with gentamicin in an MRSA mouse thigh infection model, which mimics human deep-seated chronic infections (5). Consistent with a previous study (5), vancomycin, gentamicin, or a combination of the 2 did not significantly reduce MRSA CFUs in the mouse thigh (Fig. 3C), suggesting that the infecting bacterial cells are persisters. Although 30 mg/kg bithionol alone showed no effect on the viability of MRSA persisters, 30 mg/kg bithionol combined with 30 mg/kg gentamicin killed >90% of the MRSA persisters (P < 0.001; Fig. 3C). We evaluated the hepatic and renal toxicity of bithionol in the mice in the experiments described in Fig. 3C by measuring serum levels of alanine aminotransferase (ALT) and blood urea nitrogen (BUN) (SI Appendix, Fig. S8). Although the combination of vancomycin and gentamicin significantly increase BUN levels (P < 0.01), the combination of bithionol and gentamicin increased neither.

**The Killing of MRSA Persisters Is Correlated with Increased Membrane Fluidity.** The SAR studies reported here show that some compounds that permeabilize MRSA persister cell membranes to SYTOX Green, such as Bitin-S, BT-OF, or BT-pF, do not kill them (Table 1 and SI Appendix, Figs. S6 and S7). We reported a similar result in a previous SAR study on nTZDpa (12), where we observed that nTZDpa-anaIogues 6 and 11 (Fig. 4 B and C) induced rapid SYTOX Green permeabilization, but did not affect the viability of MRSA persisters (12).

Nevertheless, the bithionol SAR results showed that only compounds leading to a significant increase in membrane fluidity killed MRSA persisters (Fig. 4A, Table 1, and SI Appendix, Figs. S6 and S7). To test the hypothesis that there is a general correlation between an increase in membrane fluidity and antipersister potency, we measured the effect of nTZDpa and 11 nTZDpa derivatives (Fig. 4 B and C) (12) on MRSA membrane fluidity. Consistent with the bithionol analogs, only nTZDpa analogs that exhibit antipersister potency showed increased membrane fluidity at 32 μg/mL. Furthermore, we observed substantial correlation between the concentration of compound required for persister killing and the amount of induced membrane fluidity as measured by Laurdan GP for all 21 tested compounds at 32 μg/mL (R = 0.64, P of slope < 0.0001; SI Appendix, Fig. S9). Consistent with these data, a recent study reported that the antibiotic agent rhodomyrtone causes increased membrane fluidity in Bacillus subtilis and kills its persister cells (23).

The data in this section show that the killing of MRSA persisters is achieved only when membrane damage by membrane-active agents is sufficiently severe to show increased membrane fluidity, potentially making membrane fluidity a biophysical indicator to identify and measure the potency of antipersister antimicrobial agents.

**Discussion**

Membrane-active agents have attractive properties as antimicrobial agents, including fast killing rates, antipersister potency, synergism with other antibiotic agents, and a low probability of
resistance selection (7). Unfortunately, most of these agents also indiscriminately disrupt mammalian membranes. However, evolution has taken advantage of differences in bacterial and eukaroytic membranes, as reflected in the production of cationic antimicrobial peptides by animals and plants that specifically target bacterial cells. Gram-positive bacterial membrane lipid bilayers contain ∼25% anionic phospholipids, whereas mammalian membranes are composed of zwitterionic (neutral) phospholipids and 20 to 50% cholesterol (27, 34). Cationic antimicrobial peptides bind preferentially to negatively charged bacterial membranes, and cholesterol in animal membranes creates a condensing effect that confers membrane rigidity and prevents the penetration of antimicrobial peptides (34).

We found that bithionol penetration into negatively charged bacterial mimetic lipid bilayers (7DOPC/3DOPG) is energetically favorable, whereas bithionol penetration into cholesterol-rich mammalian mimetic lipid bilayers (7POP/3cholesterol) is energetically unfavorable (Fig. 2A and B). Further, as the proportion of cholesterol increases from 0 to 30%, the penetration of bithionol becomes increasingly unfavorable (SI Appendix, Fig. S34), indicating that cholesterol plays a key role in bithionol’s membrane selectivity.

MD modeling suggests that the 2 polarized phenolic hydroxyl groups of bithionol play a major role in the presumed initial binding to phospholipid headgroups via hydrogen bonding. SAR studies showed that a methoxy analog of bithionol nullified bioactivity, supporting this proposed mechanism of interaction (Table 1 and SI Appendix, Table S2 and Fig. S6). In addition to providing presumed polar interactions with lipid headgroups, the bithionol choline moiety also appears to play a key role in lipid bilayer perturbation. Briefly, after attachment, the binding affinity of bithionol is dominated by hydrophobic interactions between its aromatic rings and the hydrophobic tails of the membrane lipids, which drives the penetration of the chlorinated benzene into the outer leaflet of the lipid bilayer, causing lipid bilayer perturbation (SI Appendix, Fig. S2A and C). Accordingly, the replacement of chlorine with fluorine resulted in a decrease in antimicrobial potency (Table 1 and SI Appendix, Fig. S7). Replacement of the chlorines with bromines decreased initial binding to the lipid bilayer (SI Appendix, Table S2), but increased destabilization of the membrane compared with chlorine and fluorine after penetration. Collectively, the antimicrobial activity of bithionol can be modulated by binding affinity to lipid head groups, penetration depth, and molecule size.

In contrast to bithionol and other membrane-active compounds, we have studied, daptomycin does not induce rapid SYTOX Green membrane permeabilization or kill MRSA persisters (Fig. 1C and SI Appendix, Fig. S1C). Importantly, we discovered that bithionol, nTZDpa, and their analogs that exhibit anti-MRSA persister potency induce SYTOX Green membrane permeabilization and an increase in membrane fluidity (Fig. 4, Table 1, and SI Appendix, Figs. S6 and S7). These data are consistent with previous reports that show that insertion of compounds into membrane bilayers can increase membrane disorder and fluidity, which subsequently causes passive membrane permeabilization (21, 22). Interestingly, we found that the initiation of SYTOX Green membrane permeabilization occurs at a lower concentration of the membrane-active compounds than is required to increase membrane fluidity (Fig. 2 D and E and SI Appendix, Figs. S6 and S7). Furthermore, some membrane-active agents, such as bitin-S, bithionol fluorine analogs, and nTZDpa-analogs 6 and 11, that induce SYTOX Green membrane permeabilization do not cause a change in membrane fluidity and do not kill persisters (Fig. 4 B and C and SI Appendix, Figs. S6 and S7). Bacterial membranes consist of lipid rafts organized into microdomains having different lipid compositions (35). Depending on lipid compositions, one domain may be less ordered and more fluid, whereas another domain may be more ordered and rigid (35). Because ordered and rigid domains show more resistance to membrane active agents (35) up to a certain threshold concentration, only less ordered domains would be affected by membrane-active compounds, thus making them SYTOX Green-permeable. However, this type of
localized membrane damage may not be sufficient to cause an overall increase in membrane fluidity. Over the threshold concentration, most membrane domains would be disrupted, and, subsequently, overall membrane fluidity would increase. It is also possible that some compounds may attack only less ordered and more fluid areas of membranes, which causes SYTOX Green membrane permeability, but not an overall increase in membrane fluidity. In any case, the killing of MRSA persisters is apparently achieved only when the bacterial membrane is sufficiently damaged to show increased membrane fluidity as detected by Laurdan GP.

In conclusion, we report that the clinically approved anthelmintic bithionol is an effective antimicrobial agent against both multidrug-resistant and persistent Gram-positive pathogens. Bithionol kills Gram-positive (but not Gram-negative) bacterial cells by disrupting lipid bilayers while maintaining high selectivity for bacterial compared with mammalian membranes, a consequence of the presence of cholesterol in mammalian membranes. Further, bithionol in combination with gentamicin effectively eradicates S. aureus persisters in vitro and significantly reduces bacterial burden in a mouse model of chronic deep-seated MRSA infection. We also demonstrate that increased membrane fluidity is a biophysical indicator to identify potent anti-persister compounds. Our studies provide further understanding of the molecular mechanisms by which membrane-active small molecules selectively disrupt Gram-positive bacterial membranes and support the conclusion that membrane-active antimicrobial agents have promising potential to be used for treating chronic infections caused by bacterial persisters.

Materials and Methods

SI Appendix, Materials and Methods describes in detail the materials and procedures used in this study, including bacterial strains and growth conditions, antimicrobial agents and chemicals, transmission electron microscopy, a minimal inhibitory concentration (MIC) assay, a killing kinetics assay, a persister cell killing assay, a biofilm persister killing assay, a bacterial membrane permeability assay, a mammalian membrane permeability assay, a membrane fluidity assay, a human blood hemolysis assay, a giant unilamellar vesicles (GUVs) assay, all-atom molecular dynamics (MD) simulations, a deep-seated mouse thigh infection model, and general procedures for the synthesis of bithionol analogues.

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