Using Membrane Perturbing Small Molecules to Target Chronic Persistent Infections

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ARTICLE

Using Membrane Perturbing Small Molecules to Target Chronic Persistent Infections

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After antibiotic treatment, a subpopulation of bacteria often remains and can lead to recalcitrant infections. This subpopulation, referred to as persisters, evades antibiotic treatment through numerous mechanisms such as decreased uptake of small molecules and slowed growth. Membrane perturbing small molecules have been shown to eradicate persisters as well as render these populations susceptible to antibiotic treatment. Chemotype similarities have emerged suggesting amphiphilic heteroaromatic compounds possess ideal properties to increase membrane fluidity and such molecules warrant further investigation as effective agents or potentiators against persister cells.

Introduction

The need for antibiotics with novel mechanisms of action has been a mounting problem for over a decade. In 2014, O'Neill released a mathematical analysis that predicted that antibiotic resistant infections may be the leading cause of death by 2050 if the situation remains unrectified.[1] In addition, the United States Centers for Disease Control and Prevention (CDC) found in their 2019 review that over 2.8 million antibiotic resistant bacterial infections occur per year in the United States alone.[2] Additionally, the CDC found several urgent threat bacterial species, many of which are members of the ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter* species) that are known for their multi-drug resistance.[3] This situation is more dire as there has been a dearth of new antibiotics brought to market over the last 20 years.[4] The lack of novel commercialized antibiotics is a compounded issue involving strenuous approval processes from the United States Food and Drug Administration (FDA) as well as the lack of financial support from pharmaceutical companies.[5–7] However, there is a larger antagonist: the constantly evolving bacteria.

Antibiotic compounds existed long before human interference and are utilized by bacteria to ward off competing microbes.[8] Over time bacteria have been able to develop mechanisms to avoid the killing action by these natural products, which has translated to the development of resistant mechanisms against antibiotic drugs. Often, these mechanisms occur within a few years of when a therapeutic is approved; therefore, extensive research into the evolution of resistance is required prior to a drug's approval.[9] Bacterial resistance is the summation of genetic adaptations that occur either through alteration of the genome *via* random point mutations, or through targeted alterations to the drug's biological target, thereby disrupting its mechanism of action (MoA).[10–14] In addition to genetic mutations, bacteria can also obtain resistant genes through mobile genetic elements *via* horizontal gene transfer from a nearby resistant organism.[15,16] These alterations to the bacterial genome can select for resistance through several mechanisms including structural modification of the drug target causing disruption of binding, upregulation of efflux pumps to remove the toxic compound, and drug-modifying enzymes rendering them ineffective, among others.

Though resistance is often the focus when discussing antibiotic development, bacterial persistence is a more elusive mechanism utilized by cells to avoid the killing power of antibiotics. Different from resistant bacterial populations, persister cells are genetically identical to wild type, but phenotypically dissimilar.[17,18] These stochastically formed cells are present in most bacterial cultures at <1% population density.[19] They are often referred to as "dormant" and "metabolically inactive," thereby evading common antibiotic mechanisms that rely on growth dependent processes (i.e., DNA gyrases, membrane phospholipid synthesis, etc.) as well as metabolically active targets (i.e., ATP-dependent enzymes, active efflux, etc.).[20] These cells do not proliferate during antibiotic treatment, but rather once the pressure is removed. They can switch back to a growth state, which can lead to chronic infections. Hence, the ability to target these cells has been a major area of research for inhibitor development.

Although it is desirable to target proteins or enzymes to ensure increased selectivity of killing bacterial cells, most common targets are inactive in persister cells. Conversely, an underutilized MoA is targeting the structural integrity of the bacterial membrane as this is essential regardless of growth and

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metabolic activity.[21] Within the past decade, several small molecules have been uncovered that perturb the membrane of both wild-type and persister cells of Gram-positive and Gram-negative bacteria. Through these studies a common chemotype has emerged, the inclusion of phenolic functional groups. In our analysis, we have also found that heteroaromatic structures further decorated with nitrogen based functional groups proved to increase activity in perturbing Gram-negative bacteria. Within this review, we present a focused group of small molecules that perturb bacterial membranes as a mechanism to target persister cells. Additionally, we explore common motifs, or chemotypes, of these small molecules to propose new avenues for antibiotic development. This review specifically highlights compounds that emulate the potential for future development as inhibitors or potentiators of bacterial/persister cells.

Tolerance and Persistence

Although resistance is often discussed as the major hurdle facing the development of antibiotics, another daunting issue overlooked is tolerance. The well-known, resistant bacteria are characterized by genetic mutations that give cells the ability to grow in the presence of high concentrations of antibiotics, thereby increasing the minimum inhibitory concentration (MIC) of the therapeutic.[18,22] Conversely, tolerance is the ability of cells to temporarily evade or delay death in the presence of high doses of bactericidal antibiotics without altering the MIC.[23–26] Therefore, in comparison to resistant bacteria, tolerant bacteria require longer treatment times rather than higher concentrations of antibiotic. This archetype is achieved through slowed growth and reduced metabolism, which is induced through environmental stress. By slowing or halting these processes, the cells can avoid the bactericidal action of most antibiotics *via* reduced cellular uptake as well as decreased target activity as most biological targets involve metabolism, growth, etc. Therefore, tolerant bacteria contribute to treatment failure.

The broad category of tolerance is a whole-population characteristic. Within this umbrella is a subcategory known as persistence, which is a subpopulation phenomenon and exists in most bacterial cultures at a 0.001 to 1% population density.[19] Though these numbers are small, these antibiotic evading cells can repopulate an infection leading to chronic illness. The term "persisters" was originally coined by Joseph Bigger in the 1940s when he discovered that a subpopulation of *Staphylococcus pyogenes* cells proved to be unaffected and actually viable after treatment with penicillin.[27] This novel concept has been reconfirmed through several studies.[17,28–30] In addition, research over the past decade has focused on understanding how these cells form, their biological ramifications, and most importantly how to eradicate these elusive cells. These topics have been extensively reviewed previously.[17,20,39,31–38]

Bacterial persisters are "slow-growing or growth arrested cells that have a decreased susceptibility to … bactericidal antibiotics within an otherwise susceptible clonal population."[40] These cells can be distinguished from other tolerant bacteria by their time-kill curves.[41] Bacterial populations containing persister cells have a "biphasic" killing curve (Figure 1B). This two-part feature comes from an initial steep drop in concentration of the susceptible bacterial cells after the induction of the antibiotic stressor followed by decreased killing kinetics from the persister cells.[41,42] In contrast, tolerant cells are characterized by slowed, but linear killing times in comparison to susceptible populations. To mark these differences, the minimum duration for killing (MDK) is used rather than the MIC because the MDK of a certain antibiotic against a strain of bacteria is different for persistent, tolerant, and resistant cultures. Additionally, persister killing concentration (PKC) is also used in place of MIC to distinguish between persister and wild-type assays. However, most studies rely primarily on MIC data to evaluate the efficacy of small molecules, which can limit identification of small molecule therapies for persistent populations.

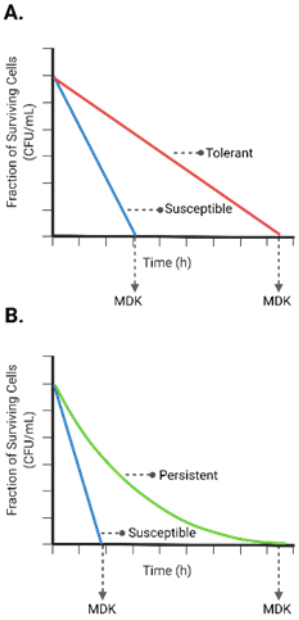


Figure 1. (A) Comparison of time-kill curves of tolerant cells (red) to a susceptible population (blue). Tolerant cells require longer treatment times. (B) Comparison of time-kill curves of persistent cells (green) and susceptible populations (blue). Persistent cells have a biphasic time-kill curve. Figure was made using BioRender.

Part of what makes persister cell research particularly challenging is the lack of universally accepted gene expression to account for phenotypic variation of the persister cell trait.[29,43,44] High-throughput screening methods traditionally used to measure gene expression require high purity samples. Alas, obtaining high purity samples of persister cells, which are heterogenous and make up an extremely small portion of the bacterial population density, is a demanding task.[45] Visualization and quantification of the SOS response (i.e. the cell's global response to DNA damage) of persisters in a

bacterial population has been accomplished by coupling microfluidics to a fluorescent SOS reporter. Although this approach successfully differentiated persisters and monitored phenotypic changes of single cells during slow growth, in depth investigations of changes in gene expression remain underexplored.[46] Fluorescence activated cell sorting (FACS) with flow cytometry has also been utilized to isolate persister cells, but high-purity samples are often unobtainable.[47,48] Strategies to chemically induce dormant or slow growing states such as treatment with *m*-chlorophenylhydrazone (CCCP)[49] have been shown to increase persister cell numbers in *Escherichiacoli*[50]*, S. aureus,* and *P. aeruginosa* and may be used in conjunction with microfluidics in the future to eliminate the need for separation techniques and gain a better understanding of the role of protein expression in heterogenous persiter formation.[51] Furthermore, advances in DNA and RNA sequencing, in particular the sensitivity of RNA-seq, will undoubtedly aid in real-time analysis of persister induction.

Current research capitalizes on environmental stressors that have been shown to increase the subpopulation to observable quantities.[52] These investigations are clinically relevant because stress-induced persister cells have demonstrated the ability to survive exposure to antibiotics in a dormant state that have been linked to chronic infections.[25,42,53] After initial clearance of the susceptible population, persister cells lie dormant. Upon completion of antibiotic course, persister cells may return to a growing, susceptible state, which can reconstitute infections (Figure 2). Although studies have commonly assumed persisters are metabolically dormant populations, there is growing evidence to suggest additional active mechanisms of persistence exist such as increased expression of efflux pumps or decreased intake of antibiotics.[54] Developing robust methods and best of practice procedures to study persistence will aid in elucidating and understanding these various mechanisms.

There are well-documented environmental stressors that increase persister cell concentrations including antibiotic exposure, nutrient deficiency, hypoxia, and oxidative stress, among others.[50,55–57] These types of persister cell formation are termed "triggered persistence."[41,52] There is also "spontaneous persistence," which occurs when a bacterial culture is at a steady-state exponential growth. In these conditions, persister cells form stochastically and remain constant if the growth conditions stay the same. However, triggered persistence is more common.

Perhaps the biggest detriment that arises from persister research is the replicability of studies.[58] It is critical that careful attention be paid to experimental design when assessing results. Regarding antibiotic-induced persistence, there are four focuses that should be acknowledged to differentiate persister cells from tolerant and resistant cells.[41,59] First, studies should attempt to re-inoculate any surviving bacteria from the last portion of the kill assay to replicate the same biphasic curve. This is to ensure that the slow growth is not due to resistant cells because persister cells intrinsically show the same response to antibiotic exposure. Second, high concentrations of antibiotics should be utilized since resistant cell growth depends on antibiotic concentration. Conversely, the killing curve of persister cells is only weakly dependent on antibiotic MIC. Additionally, prophages expressed in response to stress have been shown to decrease persister cell numbers at lower concentrations of antibiotics and can interfere with results from subsequent bioactivity assays.[60] Third, media conditions and antibiotic selection should be closely monitored to ensure that drug degradation or accidental starvation do not contribute to persister cell formation. Lastly, studies should note the conditions that occur after antibiotic exposure is removed and cells are allowed to recover. Because persister research should be dependent on time-kill assays, appropriate time should be given for the completion of the biphasic curve to emerge. Previous studies that have been conducted have ranged from 5 hours[58] to upwards of 24 hours.[27,46,61]

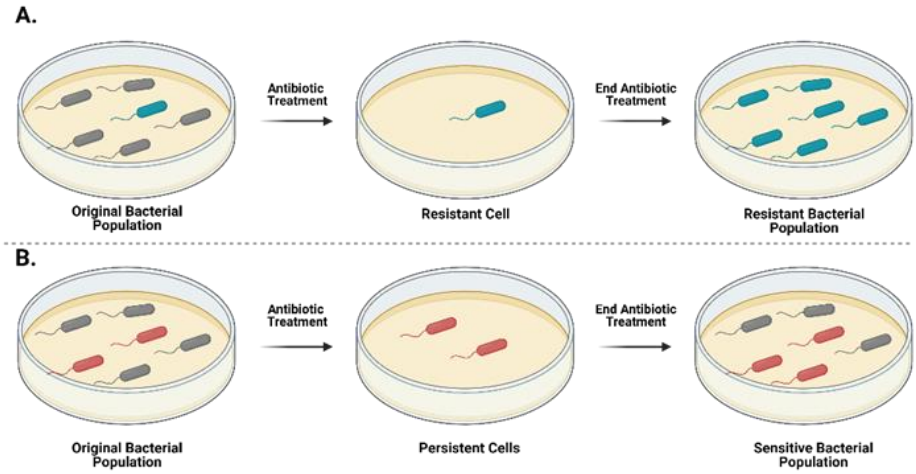


Figure 2. A) Resistant bacterial culture exposed to antibiotic. Blue cells represent resistant cell. B) Persistent bacterial culture exposed to antibiotic. Red cells represent persistent cells. Gray cells represent wild-type cells. Figure was made using BioRender.

Targeting Persister Cells *via* Small Molecule induced Membrane Perturbation

Not only do many challenges exist in the biological evaluation of the presence and study of persisters, but it is also difficult to evaluate the potential of small molecules in targeting persister cells. As our understanding of persister development has evolved over the past few decades, many have proposed targeting particular genes, proteins, etc. that appear to play a role in their development.[32,36,38,62–65] This becomes difficult as persister cells often have decreased production of uptake machinery on their outer membrane, which can reduce the ability of antibiotics to access the cell. Additionally, the possibility of resistance development is much higher when targeting a particular protein or gene.[66] Because of this, Hurdle, *et al.* (2011) proposes that a more profitable mechanism of action to target persister cells would be membrane perturbation.[21]

Membrane perturbation is a well-studied MoA in antibiotic research; however, it is often avoided by scientists due to potential off-target effects and toxicity. Though this is possible, there are several drugs currently on the market that utilize membrane perturbation as a mechanism of killing including nisin,[67,68] daptomycin,[69] polymyxin B,[70,71] and colistin (Figure 3).[72] When molecules target the membrane of bacterial cells, they generally do so in two ways.[21,73–75] First, small molecules can act through permeabilization mechanisms in which the compound induces small pores or other destructive actions to the membrane structure. This can lead to increased permeability of other small molecules to enter the cell as well as potential for leakage of cell machinery and nutrients—ultimately leading to cell death.[76,77] The second mechanism is depolarization of the membrane. Through this mechanism, compounds can cause disruptions in the electronic gradient of the bacterial membrane *via* formation of ion-conducting pores, increasing ion-permeability or by acting as an ion carrier.[78] Each of these pathways ultimately affect the proton motive force of the bacterial cell, which drives ATP synthesis and other

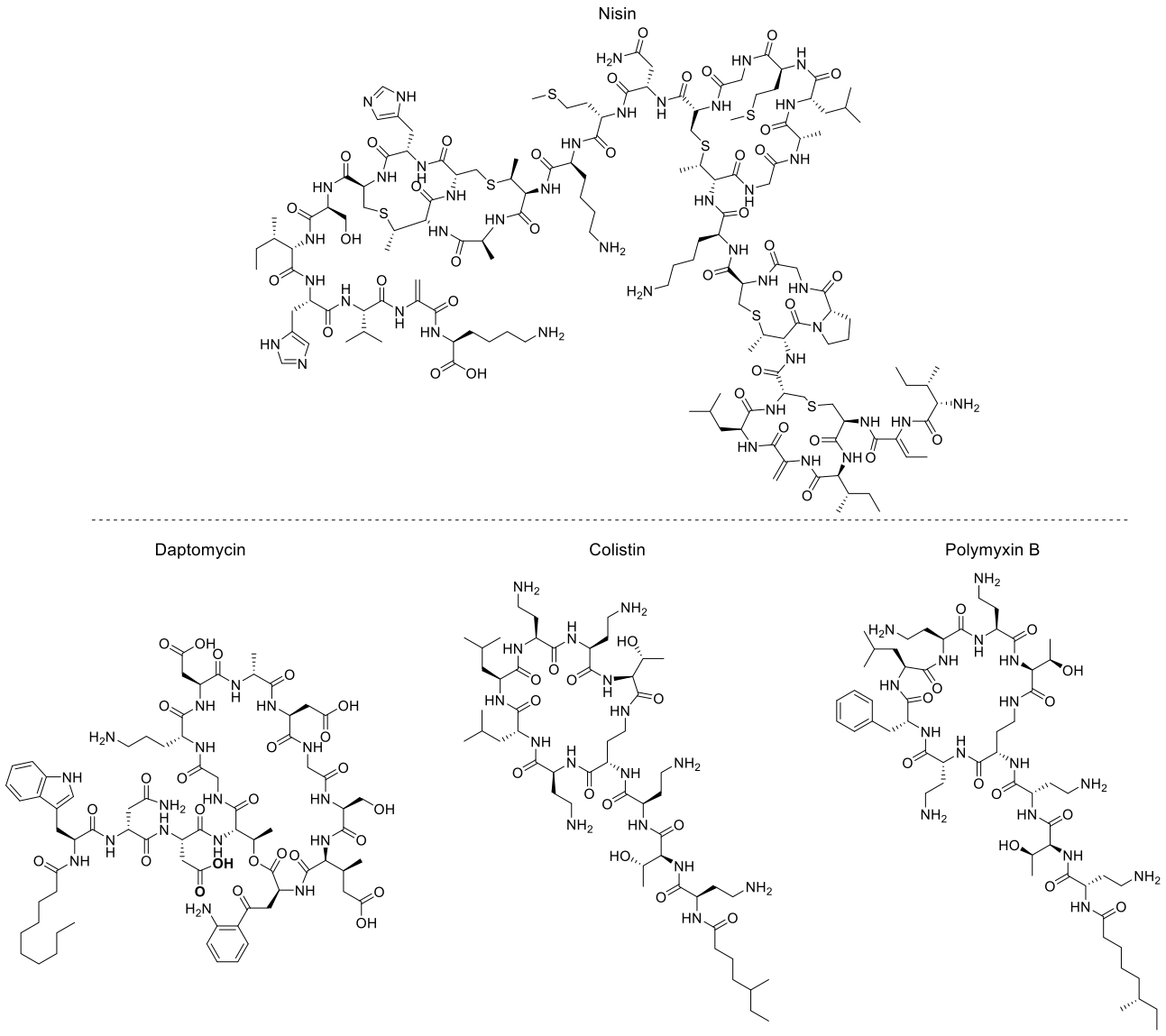


Figure 3. Chemical structures of daptomycin, colistin, nisin, and polymyxin B.

transporters across the membrane leading to either cell death or allowing for other molecules to execute killing action.[79] Assessing membrane permeability as a potential MoA can be directly studied with fluorogenic membrane dyes including Laurdan GP[80] and DilC12[81]. Of note, both dyes are heteroaromatic compounds. SYTOX Green, a nucleic acid stain, is amenable to high-throughput screens to identify small molecule membrane permeabilizers.[82] Computational investigations using molecular dynamic simulations have also been insightful in the study of small molecule-membrane interactions, in particular small molecules that demonstrate selective activity for bacterial cells.[83–85]

Regardless of growth or metabolic activity of a bacterial cell, the membrane is essential to the cell's survival. The membrane not only maintains the integrity of the cell's machinery, but it also regulates the influx/efflux of necessary nutrients. In addition, one third of the cell's proteins are located within the membrane.[75] These proteins are associated with essential cell processes such as active transport of nutrients, the expulsion of waste, and the aforementioned proton motive force that is associated with respiratory enzymes. These processes may be slowed within persister cells but are still active. Therefore, through either the permeabilization or depolarization mechanisms mentioned above, small molecules can cause lethal defects to persisters. Thus, membrane perturbation may be the "magic bullet" in targeting persister cells.

Potential of Phenol and Aryl Compounds as Membrane Perturbers for Targeting Persister Cells

The natural sources of polyphenols and aryl compounds have been a place of inspiration for antibiotic development for decades with one of the largest classes being flavonoids. A recent review by Wąsik and co-workers in 2018 highlighted the different structural classes of phenolic compounds involved in antibacterial activity against *Staphylococcus aureus.*[86] These natural products often contain a phenyl or benzyl moiety further decorated with hydroxyl or other polar groups (halogens, amines, etc.). They can also be conjugated to other aryl structures or hydrophobic alkyl chains. Through years of research into the antibacterial properties of these compounds, many have found that they possess the ability to permeabilize the bacterial membrane leading to cell death as well as opening the door for synergistic therapies with other antibiotics as explored by Jeon and co-workers in 2015.[87] Though most of these compounds have only been analyzed against wild-type cells, we hypothesize that they would also have activity against persister cells due to other successes as discussed below, though further analysis is required.

Prenylated Phenolic Compounds

A common tradition for centuries is the utilization of various plants as therapeutics. Through further analysis of the components of these plants, researchers uncovered therapeutic agents in these traditional medicines. Through this lens, Gruppen and co-workers turned to legumes, a large plant family known to produce antimicrobial compounds—particularly prenylated phenolic compounds under stress induced conditions.[88] Through flash pool collection, the authors

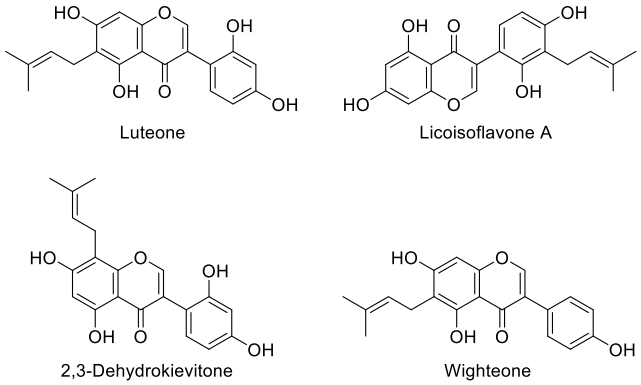


Figure 4. Examples of prenylated compounds attenuated in active fractions.

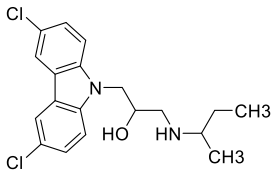
grouped 57 different phenolic compounds, 39 of which were prenylated and could be identified within the classes of isoflavonoid, flavonoid, stilbenoid, phenolic acid, and chromone.

Overall, the authors showed potent activity of the pooled compounds against *Listeria monocytogenes* with the most potent compound having an MIC of 10 µg/mL. Additionally, the compounds could elicit inhibitory activity against *E. coli* when co-administered with an efflux inhibitor. They also confirmed through further analysis that the prenylated compounds in each fraction (e.g.: luteone, licoisoflavone A, 2,3-dehydrokievitone and wighteone) were responsible for the antibacterial activity (Figure 4). To elucidate the MoA, the authors performed a membrane permeabilization assay with propidium iodide, which showed that the prenylated compounds were able to rapidly permeabilize the membrane of *L. monocytogenes* at the MIC. The authors hypothesized that the prenylated compounds intercalated into the lipid bilayer thereby disrupting its packing density and increasing permeability. Additionally, the authors proposed that structural features such as a bent skeleton confirmation, prenylated chains, and increased hydrophobicity contributed to potent antibacterial activity due to heightened perturbation of the membrane.

N-alkylated 3, 6- dihalogenocarbazol 1-(sec-butylamino)-3-(3,6-dichloro-9H-carbazol-9-yl)propan-2-ol) (SPI031)

In 2016, Michiels and co-workers identified a novel compound they named SPI031, which had potent broad-spectrum activity, including clinically relevant pathogens *S. aureus* and *P. aeruginosa*, with MICs ranging from 4.63 to 18.5 µg/mL (Figure 5).[89] This complex carbazole had similar killing kinetics to that of polymyxin B, a last line of defense antibiotic, as well as improved kinetics in comparison to vancomycin against *P. aeruginosa*. Through SYTOX Green assays, the

researchers identified that SPI031 perturbed the membrane of methicillin-resistant *S. aureus* cells as well as the inner and outer membranes of *P. aeruginosa* exhibited by the cellular uptake of the fluorescent probes. This perturbation was further confirmed *via* phospholipid mimicking liposomes filled with carboxyfluorescein (CF) fluid. After treatment with SPI031, an increase of CF leakage was observed in comparison to negative controls.



SPI031

Broad Spectrum Activity
MIC= 4.63 to 18.5 μg/mL

Figure 5. Chemical structure of *N*-alkylated 3, 6- dihalogenocarbazol 1-(sec-butylamino)-3-(3,6-dichloro-9H-carbazol-9-yl)propan-2-ol*)*, SPI031.

In addition to these results, the authors also studied potential resistance mechanisms through whole-genome sequencing of spontaneous resistant mutants of *P. aeruginosa*. This analysis revealed mutations in multidrug efflux pumps as well as genes involved in outer membrane synthesis. They hypothesized that alteration of the outer membrane structure was able to confer resistance to SPI031. The authors suggest that this compound may be a very potent antimicrobial especially against persister cells, though these studies were not performed. However, they do point to potential toxicity issues due to targeting of human keratinocytes and human hepatoma cells, which is often a point of contention for most membrane targeting small molecules. The authors suggest that further structural modifications to the scaffold may lead to improved specificity towards bacterial membranes over human.

1-((2,4-dichlorophenethyl)amino)-3-phenoxypropan-2-ol (SPI009)

Following the study published in 2016, Michiels and co-workers identified another compound they named SPI001 through a high-throughput screen of over 20,000 small molecules in 2017.[90,91] The compound displayed potent activity in combination with 10 μg/mL of ofloxacin against persistent *P. aeruginosa* cells, with a >2,000-fold reduction in comparison to ofloxacin alone. With this initial hit, the researchers performed structure-activity-relationship (SAR) studies and found a more potent analog: 1-((2,4-dichlorophenethyl)amino)-3-phenoxypropan-2-ol, or SPI009 (Figure 6). At 68 μg/mL, SPI009 proved to have a 7,200-fold reduction in *P. aeruginosa* persister cell concentration in comparison to ofloxacin alone. To further analyze the killing capability, they treated isolated persister and non-persister cells at a range of concentrations from 17 to 68 μg/mL of SPI009 alone. This study suggested that SPI009 could kill both persister and wild-type cells. Additionally, Michiels and co-workers displayed that SPI009's combination therapy was not only limited to ofloxacin, but also proved potent in combination with amikacin and ceftazidime.

After analyzing the compound's biological activity, the researchers turned to further understanding its MoA. They began with a *P. aeruginosa* knockout library to identify cells that had decreased sensitivity to the SPI009 and ofloxacin treatment. This showed an over-expression of genes generally involved in adaptation and protection as well as cell wall and

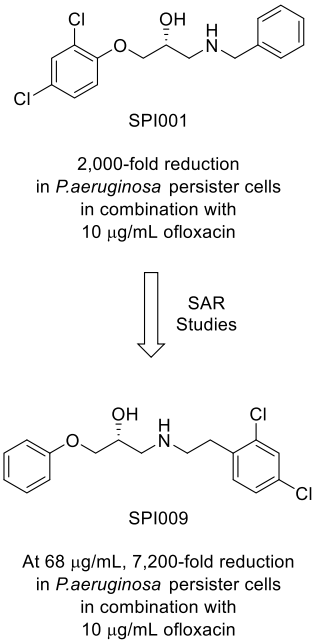


Figure 6. Chemical structure of the parent scaffold, SPI001, and the most potent analog (1-((2,4-dichlorophenethyl)amino)-3-phenoxypropan-2-ol (SPI009).

lipopolysaccharides (LPS) synthesis and maintenance. Overall, these data suggested that membrane integrity was the target. To confirm these findings, they performed a macromolecular synthesis assay, which confirmed the ability of SPI009 to reduce the incorporation of precursors for DNA, RNA, proteins, fatty acids, and peptidoglycan. To analyze its ability to damage the membrane, they performed artificial bilayer and permeabilization studies in addition to analyzing treated cells under a microscope. The artificial membrane displayed increased CF leakage in comparison to an inactive analog. Additionally, the permeabilization assay with SYTOX Green showed that SPI009 targeted the inner and outer membrane, which was further confirmed through microscopic analysis. Altogether, these findings indicate that SPI009 can disrupt both the outer and inner membrane of *P. aeruginosa* persister cells as well as inhibit macromolecular synthesis. Through their findings, the authors propose that SPI009 would be a good therapeutic alone as well as in combination with outdated antibiotics that are deemed unusable due to decreased sensitivity.

Substituted Indoles

Previous misconceptions that indole—an intra-species, inter-species, and interkingdom signal molecule—is responsible for an increase in persistence in various bacteria, have recently been widely disproven.[92] In fact, many recent studies have demonstrated the opposite, and it can now be said that

substituted indoles may have significant potential against bacterial persister cells. Several studies have come to prove that substituted indoles are capable of killing dormant cells and decreasing persistence by disrupting the cell membrane.[93]

Amphiphilic Indole Derivatives

In 2017, Yang and co-workers performed an extensive SAR study and biological evaluation of 1-geranylindole (Figure 7).[94] Previously, the authors had discovered this compound in a focused screen of indole-type small molecules. This particular indole caught their interest as it had low micromolar activity with a minimum inhibitory concentration required to reduce bacterial growth by 50% (MIC_{50}) of 5 μM against *Mycobacterium bovis* and MIC_{50} of 3.5 μM against *Mycobacterium tuberculosis*. Additionally, 1-geranylindole showed membrane perturbation, which was attributed to its amphiphilic nature. Though 1-geranylindole offered promise as a potent therapeutic, the authors found that it had toxicity against mammalian cells. To improve this issue, the authors sought to perform an extensive SAR study with five different series of analogs. These analogs explored the four different functionalities of 1-geranylindole: (1) role of the side chain at the indole nitrogen; (2) basic N-substituted aminomethyl side chain; (3) 5-fluoro substituent; and (4) replacing the indole with its isosteric equivalent, 7-azaindole. The fifth series of analogs was the compilation of the best structural changes from the previous series that offered improved selectivity towards the mycobacterium.

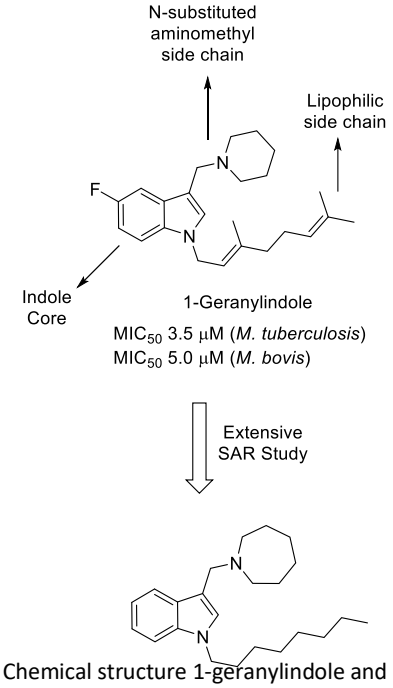


Figure 7. Chemical structure 1-geranylindole and potent analog, 74a.

Through extensive analog development, the authors found that the lipophilic side chain at the indole nitrogen and the basic N-substituted aminomethyl side chain were required for activity. The other components of the molecule were otherwise amenable without significant change to its activity. Of the library of analogs synthesized and evaluated, analog 74a was ultimately selected due to its low micromolar activity (MIC_{50} = 2 μM), selectivity for *M. tuberculosis*, therapeutically relevant solubility, and *in vitro* metabolic stability. With this compound, the authors sought to further explore its MoA, namely its membrane perturbation capabilities. This was achieved by monitoring both the membrane potential and permeability. The membrane potential was measured with 3,3-diethyloxacarbocyanine iodide, which fluoresces red in polarized cellular membranes and green when membranes are depolarized due to lack of intracellular accumulation. 74a showed a time-dependent decrease of membrane polarization at 2 × MIC. Membrane permeabilization was measured with propidium iodide accumulation, which also showed a time-dependent increase in cell permeabilization. Additionally, 74a was able to not only inhibit growing cells of *M. tuberculosis* but also persister cells. To probe its broad-spectrum activity, the authors also explored the activity of 74a against *S. aureus* and *E. coli*. 74a retained activity against *S. aureus* with an MIC_{50} of 8 μM but was ineffective against *E. coli*. With these promising results, indole compounds offer a new avenue for novel therapeutic development.

SCH-79797

In summer of 2020, Gitai and co-workers disclosed the potent antibacterial activity of the pyrroloquinazolinediamine complex, SCH-79797 (Figure 8).[96] They targeted this compound *via* a small molecule library screen of over 30,000 unique compounds that were previously reported as human PAR-1 antagonists. SCH-79797 was previously identified as an antimicrobial by Gupta, *et al.* and had extensive studies on its *in vivo* efficacy in animal studies.[97–99] To further confirm its activity, the authors began with MIC assays, in which they discovered that the compound had potent broad-spectrum activity, including several ESKAPE pathogens with an MIC of 2 to 6.25 μg/mL.

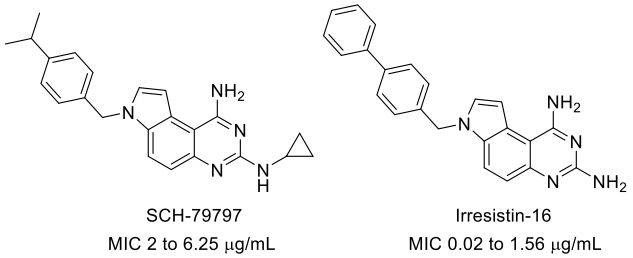


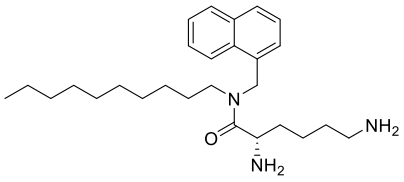
Figure 8. Chemical structure of SCH-79797 and Irresistin-16.

After failed attempts to produce resistant mutants, the authors employed a multipronged approach to unveil SCH-

79797's MoA. This began with bacterial cytological profiling, which analyzed SCH-79797 against a panel of known classes of antibiotics. Through this analysis, the authors identified that their compound had a unique MoA, which prompted the use of thermal proteome profiling, CRISPRi genetic sensitivity, and metabolomic profiling for further characterization. These analyses identified the possible targets as dihydrofolate reductase and the bacterial membrane. These were further confirmed *via* enzymatic assays as well as depolarization and permeabilization assays. Ultimately, the authors proved that SCH-79797 can simultaneously damage the integrity of bacterial membranes while also inhibiting folate synthesis through inhibition of dihydrofolate reductase. Additionally, they synthesized a simplified analog, Irresistin-16, which had increased activity as well as reduced toxicity.

Peptoid Mimics: Aryl-Alkyl-Lysines

In recent years, cationic antimicrobial peptides (AMPs) have become a large focus of antibiotic development, especially in targeting persister cells.[100,101] These compounds often mimic molecules that are a part of the innate immune system used as the body's first line of defense.[102–104] They can inflict killing by perturbing and eventually lysing the bacterial membrane.[101,105] Additionally, as the compounds are quite complex, resistant mechanisms are rare. Though the use of AMPs seems a promising target for antibiotic development, they are limited by high levels of toxicity, ease of degradation in the presence of proteases, and high cost to produce at an industrial scale. To combat these issues, Haldar and co-workers in 2014 sought to synthesize peptoid mimicking molecules in which they incorporated an L-lysine for cationic character, an aromatic core for hydrophobicity, and a varied alkyl chain (Figure 9).[106] They synthesized over 16 analogs through a three-step synthetic sequence with their best performing analog being NCK-10 (napthalene core with decyl chain appendage) with an MIC of 4.1 to 4.8 μg/mL against both wild-type and persister MRSA cells.[107]



NCK-10

MIC 4.1 to 4.8 μg/mL

Figure 9. Structure of NCK-10.

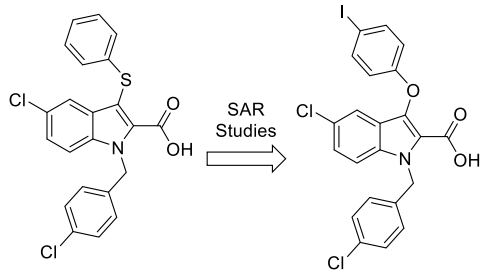
Additionally, in further analyzing the compound's activity, the authors found that NCK-10 was able to completely eradicate MRSA persister cells at 5 × MIC in 30 min. In analyzing its MoA, they uncovered that NCK-10 rapidly depolarized the membrane of MRSA persister cells within 5 min of treatment, but the membrane permeabilization appeared weaker through observation of varying fluorescence intensity in their assays. In addition to the persister activity, NCK-10 reduced the number of viable cells within a biofilm as well as reduced the mass of pre-formed biofilm at 10 × MIC. Overall, Haldar and co-workers showed the ability to simplify complex AMPs thereby producing several potent antibacterials that could be used to treat planktonic, persistent, and biofilm MRSA infections.

Repurposed FDA Drugs prove Potent in Targeting Bacterial Persister Cells

In pursuit of antibiotics targeting persister cells, the Wuest lab developed several projects focused on perturbing cell membranes. Rather than identifying new molecules, these projects explored repurposing drugs previously approved by FDA for novel antibiotic use. Through collaboration with Mylonakis's laboratory, they were able to uncover several potent small molecules *via* a high-throughput screen. This screen involved a *Caenorhabditis elegans*/ MRSA infection model, which enabled the investigation of compound potency as well as toxicity concurrently.[108,109] From this screen and further analysis, they uncovered several compounds including three small molecules (nTZDpa, CD437, and bithionol) that proved efficacious against MRSA persister cells.

nTZDpa

nTZDpa is a nonthiazolidinedione that consists of several key moieties including a 5-chloro-substituted indole core, a 1-chlorobenzyl substituent, a phenyl sulfide moiety, and a carboxylic acid (Figure 10). It has previously been investigated as a potential diabetes therapy.[110] After initial hits through the *C. elegans*-MRSA infection model, computational modeling was performed to further uncover the MoA of nTZDpa against MRSA cells.[85] This modeling showed that the MoA involved permeation of the membrane *via* high affinity of the carboxylic acid and two chlorine atoms for the phospholipid heads on the cell's surface. After association, perturbation from the aryl groups ultimately leads to cell lysis and death. Initial bioactive results showed relatively high but promising MIC of 4 μg/mL against growing cells of *S. aureus*. Additionally, a PKC was measured at 64 μg/mL. Since compounds that are effective against bacterial membranes can also be toxic to mammalian cells,[108] hemolysis against human erythrocytes were measured to



nTZDpa

MIC 4 μg/mL
PKC 64 μg/mL
HC_{50} 47 μg/mL

Analog 14.
Lead Compound

MIC 1 μg/mL
PKC 16 μg/mL
HC_{50} >64 μg/mL

Figure 10. Chemical structure of nTZDpa and the lead compound, Analog 14.

monitor toxicity with average hemolytic activity (HC_{50}) of nTZDpa measured at 47μg/mL. These measurements served as the baseline by which future analogs were compared for their efficiency against persister cells while maintaining low toxicity to improve therapeutic viability.

The diverted synthesis of nTZDpa allowed for optimization of the parent scaffold. Among these key changes include a substitution of oxygen for sulfur to improve the toxicity profile as well as the addition of iodine to the aryl ether moiety for increased potency. The current lead analog contains a 4-iodo substitution to the aryl thioether moiety that resulted in an MIC of 1 μg/mL, PKC of 16 μg/mL, and HC_{50} of >64 μg/mL (Figure 10). These changes to the scaffold have been consistent with rules of permeability for small molecules, such as a 600 Da cutoff, low number of rotatable bonds, and low three-dimensionality.[111]

In addition to the improved efficacy of nTZDpa analogs against growing and persistent MRSA cells, the original scaffold has been shown to work synergistically with other classes of antibiotics, specifically aminoglycosides such as gentamicin, tobramycin, neomycin, kanamycin, and streptomycin.[85] Since these aminoglycosides have reported resistance in clinical strains,[112] the low probability of resistance to nTZDpa shows additional promise of implementing this drug into combination therapies. This is especially important as many antibiotics, such as aminoglycosides and β-lactams, require access to the cell's cytoplasm to disrupt their respective cellular target. Therefore, the ability of nTZDpa to perturb the membrane could serve as a bypass for these antibiotics in the challenges posed by persister cells.

CD437

Another molecule uncovered in the *C. elegans*-MRSA infection model was the synthetic retinoid and analog of vitamin A, CD437 (Figure 11). After initial discovery, further analysis showed an MIC of 1 μg/mL against MRSA persister cells as well as a reasonable toxicity panel (HC_{50} = 32 μg/mL; LC_{50} = 20 μg/mL).[83] Additionally, the compound proved to have synergistic effects with gentamicin. To gain further insight into the molecule's MoA, a specific all-atom molecular dynamic simulation was performed utilizing a synthetic lipid bilayer that imitates the bilayer of *S. aureus*. From this, researchers learned that the molecule's carboxylic acid and phenol moieties allowed it to interact with the hydrophilic heads of the bilayer followed by molecular rotation and insertion of the lipophilic adamantyl group thereby perturbing the membrane. This mechanism was further supported through molecular dynamics simulations in giant unilamellar vesicle experiments. These molecules also have the potential to aggregate to induce membrane damage. However, further testing is needed to support this hypothesis.

After CD437's initial discovery in 2018, three generations of analogs have been synthesized. The first explored changes to the carboxylic acid and phenol moieties, which led to a new lead compound: a primary alcohol derivative deemed "analog 2," which had an MIC of 2 μg/mL and improved cytotoxicity (HC_{50} >32 μg/mL; LC_{50} >31 μg/mL). Unfortunately, analog 2 possessed low solubility in serum due to its high affinity for retinol binding proteins (unpublished data), thus prompting the need for further analog exploration. The second generation of analogs explored the hydrophobic adamantyl group of the parent scaffold to mimic the fatty acid tails embedded in the lipid bilayer. These data were published in 2019, and the adamantyl

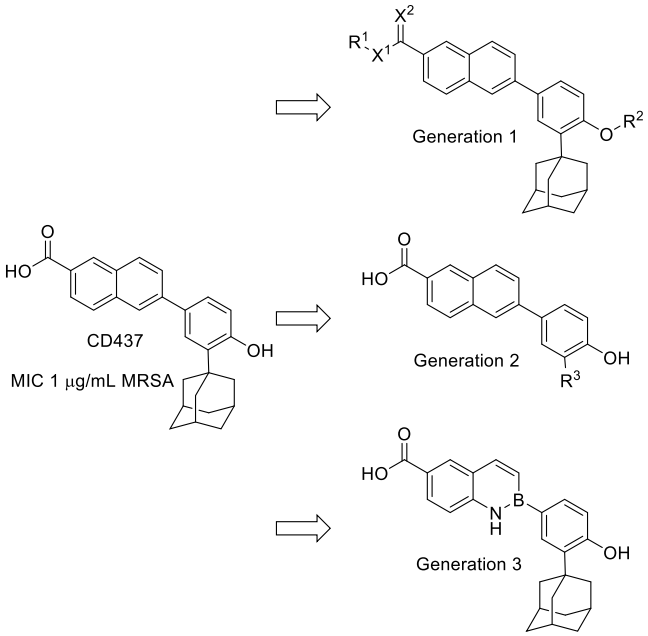


Figure 11. Chemical structure of CD437 as well as chemical representation of the analog substitutions for each generation. Generation 1: R^1 and R^2 = CH_3; X^1 = O, or NH; X^2 = O, or H,H. Generation 2: R^3 = CH_3, Ph, *tert*-butyl, or varying chain lengths of saturated or unsaturated alkyl groups.

moiety was found to have the greatest bactericidal capability.[113]

Following this generation, the authors sought to explore the broad-spectrum capability of the scaffold by attaching an alkylamine moiety to the molecule, as this addition has previously been shown to elicit activity against Gram-negative bacteria on similar substrates.[114] Unfortunately, this alteration did not gain broad-spectrum activity and decreased the potency against Gram-positive bacteria. In the latest generation, an isosteric substitution of a carbon-carbon double bond for a nitrogen-boron bond within the naphthalene scaffold was synthesized in hopes of altering the electronics enough to reduce retinol protein binding.[115] However, it was discovered that this substitution did not enhance the molecule's biological efficacy.

Bithionol

Bithionol is a chlorinated bisphenol that is clinically approved for anti-parasitic treatment of trematode infections such as *Fasciola hepatica* in equine species (Figure 12).[116] In 1965, Barr and co-workers disclosed that in addition to parasitic activity, bithionol also proved to have antibacterial activity with an MIC of ~8 to 15 μg/mL against *S. aureus*. In an effort to reproduce the results of the 1965 study, the Wuest and Mylonakis laboratories analyzed its antibacterial activity and performed SAR studies for this compound. They showed that bithionol had potent activity against several Gram-positive bacteria including the vancomycin-resistant *S. aureus* (VRSA).[84] Furthermore, they found that through their analysis it had an even lower MIC of 0.5 to 2 μg/mL than demonstrated in the previous study in 1965.

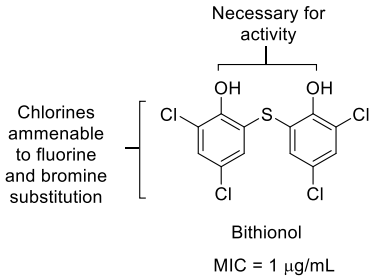


Figure 12. Chemical structure of bithionol.

In addition to preliminary bactericidal activity, bithionol also had a time-dependent reduction of *S. aureus* cell density like cetalkonium chloride, a common quaternary ammonium antiseptic, which indicated potential lytic activity. Further analysis *via* transmission electron micrographs (TEMs) confirmed this hypothesis with distorted cell membranes. This prompted further analysis of bithionol's activity against persister cells. Through analysis with MRSA MW2 strain planktonic and biofilm persisters, bithionol proved to kill these cells in a dose-dependent manner over a 2 h period, with complete eradication after 24 h exposure at 32 × MIC. Even more, it proved to have potent bactericidal activity against VRSA strain VRS1 persister cells with complete eradication at 32 × MIC.

With these results, the researchers sought to understand bithionol's MoA *via* all-atom molecular dynamic simulations. They showed that the phenol and chlorine moieties are initially attracted to the negatively charged heads of the bacterial membrane followed by penetration into the membrane, ultimately leading to membrane perturbation and cell death. This was further confirmed through biomembrane-mimicking giant unilamellar vesicle assays. Through SAR studies, the researchers showed that the phenol moieties were necessary for antibacterial activity. Additionally, substitution of the chlorine atoms with other halogens, such as fluorine and bromine, were somewhat tolerated with the latter being most active. It was hypothesized that the polar carbon-fluorine bond may increase its initial attachment to the membrane but disallows penetration further into the membrane.

Honokiol

Honokiol is a natural product isolated from *Magnolia officinalis*. Initial analysis of this compound by Wang *et al.* showed potent activity against *Streptococcus mutans*, which naturally occurs in the oral cavity and is the causative agent of dental caries.[117] Most interesting was honokiol's apparent ability to disrupt *S. mutans* biofilm. However, upon further analysis by our groups, we discovered this activity only occurred in aerobic conditions, which is not a representative environment of the oral cavity.[118] In our hands, honokiol had moderate activity with an MIC of 250 μM under biologically relevant conditions (5% - CO_2 supplemented atmosphere).

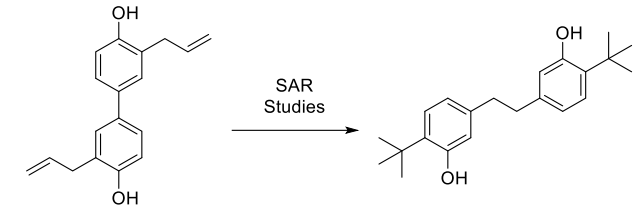


Figure 13. Chemical structures of Honokiol and Compound **C2**.

Through SAR analysis, our groups discovered our lead analog, deemed C2, that showed potent activity against *S. mutans* planktonic cells with an MIC of 2 μM (Figure 13). To further understand its MoA, resistance selection assays were first attempted against *S. mutans*.[119] However, these were unsuccessful. As it has been previously shown to be difficult to produce resistance for membrane perturbing compounds,[21] we then decided to test its membrane depolarization and lysis capabilities. C2 did not show significant depolarization in comparison to the negative control, DMSO. However, C2 did show lytic activity as evidenced by the increased propidium iodide fluorescence. This lytic activity was further visualized in TEM images. To further confirm its permeabilization activity, SYTOX Green assays were performed, which showed increasing fluorescence in the presence of C2.

With its membrane perturbing action, its minimum bactericidal concentration (MBC) was determined to investigate if C2 was bactericidal or bacteriostatic. It was shown to have an MBC 4X its MIC indicating bactericidal affects. Though the original parent compound's, Honokiol, antibacterial activity was disproven, the lead analog, C2, displayed potent activity with membrane perturbing capabilities.

Conclusions

Though bacterial resistance is frequently the focus in antibiotic development, the elusive tolerant and persister cells are often the overlooked culprit for chronic infections. Different from resistance, bacterial tolerance is a whole-population characteristic, in which the cells can evade the killing power of antibiotics without affecting the MIC of the therapeutic but rather require longer treatment times. These cells achieve this by reducing metabolism and slowing growth, which is induced

through the environmental stress of the antibiotic. To make matters worse, within the umbrella of tolerant cells are persister cells. Existing in most bacterial populations at a 0.001-1.0% concentration, persisters are a subcategory of tolerance and are distinguished from tolerant cells through their time-kill kinetics, in which they produce a biphasic curve. This is due to an initial steep drop in the killing of the susceptible population followed by a slow decrease due to the presence of persister cells.

Looking forward, we propose a few areas to focus on for future investigations. Researchers have proposed developing small molecules that target certain genes or proteins that have been shown to lead to the formation of persister cells. Though this has potential as a therapeutic strategy, these small molecules are hindered due to their decreased ability to be actively transported into the cell. It was proposed in a review by Hurdle and co-workers that another method to target persister cells would be through membrane perturbation. As membranes are essential in all cells regardless of level of growth or metabolism, utilizing this as a target for persister cells may be more fruitful. Clearly from the studies discussed above this is a viable strategy that garners more investigation.

In analyzing membrane perturbing molecules, we found that a common motif involved a polarized aryl moiety, often a phenol or indole, which was further decorated with additional hydroxyls, amines, or other halides. The group of molecules discussed in this review were discovered either in high-throughput screens of repurposed drugs or unique small molecule libraries or were simplified AMP mimics. Through analysis of the structure and activity, we propose that the membrane perturbation is generally achieved by initial attraction of the polarizable groups to the negatively charged heads of the lipid membrane. Once the compounds are associated with the membrane, the hydrophobic portions can intercalate into the membrane thereby disrupting the ordered fatty acid tails leading to cell leakage. This can either induce cell lysis causing cell death or can increase permeability of the membrane for synergistic treatment with other antibiotics. Additionally, the presence of heteroaromatic and amines groups showed broad spectrum activity, whereas phenol-based compounds were often limited to Gram-positive bacteria. Though not every study reported within this review explicitly analyzed activity against persister cells, with the success of membrane perturbation against persisters, we expect these compounds to also be successful.

Through this focused collection of membrane perturbing small molecules and identification of common chemical motifs, a proposed unifying foundation for membrane perturbation as a MoA against persister cells has been laid. This groundwork can serve as a starting point for rational design in developing novel antibiotics. As amphiphilic heteroaromatic compounds frequently displayed increased membrane perturbing effects, incorporation of these motifs to new or existing scaffolds may include this MoA. Beyond the development of novel scaffolds for membrane perturbation, there is also a need for broadening analysis of existing phenolic and heteroaromatic structures against bacterial persister cells. As discussed, it is difficult to analyze the effects of small molecules on persister populations and even ensure the presence of persisters in appropriate assays. However, as these archetypal cells can cause recalcitrant infections, there is a great need to expand upon the research of persister inhibitors in the future.

Conflicts of interest

W.M.W. is an inventor on intellectual property filed on some of the work discussed.

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