Chapter 9

Targeting Bacterial Persistence to Develop Therapeutics Against Infectious Disease

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1. Introduction

The application of the prototype antibiotics penicillin and streptomycin to bacterial infection in the 1940's marked a historic milestone in medicine and heralded a new era of antimicrobial therapy as the modern standard for infectious disease management. Yet, even in those early days of discovery, scientist Joseph Bigger noted an unexplained phenomenon. Although penicillin treatment of *Staphylococcus aureus* infection killed the great majority of the bacterial population, a small subset of cells (~1 in 10^5) continued to persist and remained recalcitrant to antibiotic-mediated killing.[1] When re-grown in the absence of antibiotic, the bacterial community once again became sensitive to antibiotic-mediated killing and resembled the original culture (~1 in 10^5 persisters), providing conclusive proof that these organisms were not drug-resistant strains that had evolved via genetic mutations (in which case all bacteria in the final population would be drug-resistant). (Fig. 1) Instead, persister cells are phenotypic variants that are genetically identical to the susceptible bacterial population, but have modified their physiology to survive environmental stress. These bacteria exhibit antibiotic tolerance that is non-heritable and reversible upon removal of the antibiotic, a completely different phenomenon than the more well-studied antibiotic resistance mechanisms mediated by genetic mutation. Persistence is akin to a community "insurance policy" in which surviving persister cells hedge against unlikely but catastrophic events, while still maintaining near optimal growth at the population level.[2, 3]

In this review, we will discuss the known molecular mechanisms that underlie bacterial persistence, the impact of persistence on infectious disease, and the different strategies that are being developed to target persisters in disease. The human toll of pathogen infection has been compounded by the rampant use of antibiotics in the last half-century, leading to the rapid evolution of drug-resistant strains to practically every approved antibiotic. There is a

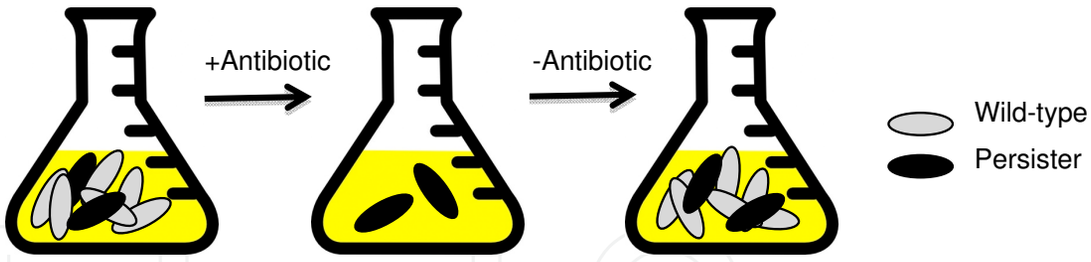

Figure 1. Bacterial persisters are recalcitrant to antiiotic killing. After removal of antibiotic, bacterial community expands to contain both wild-type and persister sub-groups, indicating that the persisters are phenotypic variants instead of containing genetic mutations.

great public health need to identify novel strategies for development of therapeutics to treat pathogen infection. Development of novel therapies that either kill persisters directly or stimulate their reversion to logarithmic growth may effectively reduce disease relapse and shorten the treatment period.[4] It may be the case that a combination therapy comprised of conventional antibiotics that kill replicating pathogens and new drugs that target the metabolically-inactive persisters can also reduce the rate of emergence of antibiotic resistance.

2. Impact of bacterial persistence on infectious disease

Without question, bacterial persistence greatly contributes to the burden of infectious disease, where persisters survive antibiotic treatment to re-infect patients in a frustrating cycle of chronic infection. Many antibiotics have been shown to be only active against dividing bacteria.[5] Persisters are thought to be dormant cells that greatly slow down essential cellular functions that antibiotics generally target, including trancription, translation, cell wall synthesis, and DNA replication. Persisters are found at relatively higher levels in stationary phase compared to logarithmic cultures, consistent with a dormant state. The persistence state has been found in many different bacterial species, including *E. coli*, *Mycobacterium tuberculosis*, and *Pseudomonas aeruginosa*, underscoring the evolution of persistence as a survival strategy in different stressful environments.[6]

There is also increasing evidence that persisters mediate drug tolerance in biofilm formation associated with chronic diseases, including endocarditis, gingivitis, and osteomyelitis.[7] Biofilms form a protective environment for persisters, shielding them from the host immune system.[8] (Fig. 2) Biofilms can form readily on in-dwelling devices, such as catheters and prostheses, or on physiological surfaces, such as *P. aeruginosa* infection of the lung in cystic fibrosis patients. The dormancy of persisters and the different pathways that lead to their formation contribute to the unique challenge in treatment of chronic infections, especially in immunocompromised patients where biofilms can form deep in the soft tissues. In addition, the presence of different subpopulations of persistent pathogens with varying antiobiotic susceptibilities further complicates treatment with optimizing drug efficiencies.

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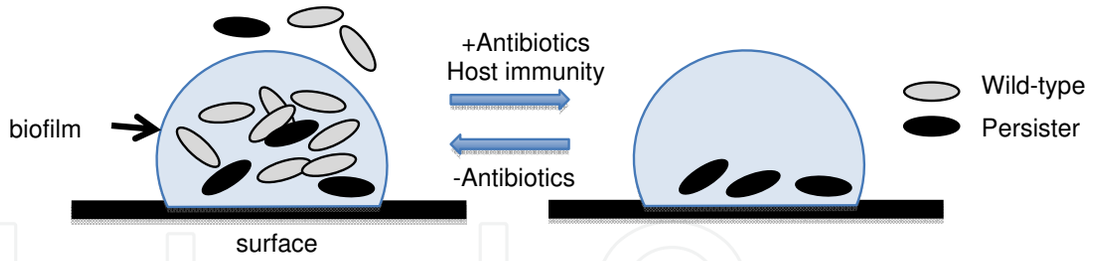

Figure 2. Biofilm formation with persisters. Biofilms can contain both wild-type replicating and persister cells. Addition of antibiotics and host immunity can kill both wild-type and persister cells in the biofilm and extracellular milieu. The biofilm matrix can protect persisters from killing and can lead to re-population of pathogen in the biofilm after antibiotics are removed.

3. Molecular mechanisms of bacterial persistence

Despite observance of the persister phenotype since the 1940's, the genetic regulatory pathways that switch bacteria into the persister phenotype remain poorly understood. Further research on bacterial persistence rapidly declined with the availability of potent antibiotics. Furthermore, there were technical difficulties in obtaining sufficient numbers of persister cells for analysis and a lack of sophisticated and sensitive methods to study rare biological events at single cell resolution. With the recent emergence of antibiotic-resistant bacterial strains, interest in the mechanisms of bacterial persistence has slowly resurged, amid rapid advances in microfluidics and advanced imaging that can be applied to single cell analysis. [9, 10] A list of genes and pathways linked to persistence is listed in Table 1.

In the 1980's, a genetic screen was performed to select for *E. coli* mutants that exhibited increased persistence in response to ampicillin exposure.[11] *hipA* (high persistence) was the first gene identified that modulated the frequency of persister formation, with the gain-of-function allele *hipA7* inducing ~1% persisters in culture, an ~1000 fold increase in persisters compared to a wild-type strain. HipA is a component of a toxin-antitoxin (TA) module that plays a role in inhibition of protein and nucleic acid synthesis in response to stress. HipA functions as a kinase that phosphorylates the essential translation factor Elongation Factor Tu (EF-Tu) to inhibit translation.[12]

The increased level of persister cells has led to the use of *hipA* mutants in multiple studies on bacterial persistence, including integration of microfluidics and single cell microscopy[13] and microarray analysis.[14] These types of studies can enable quantitative analysis of single cell behavior and gene expression dynamics to measure cell-to-cell heterogeneity in a clonal cell population. For example, growth patterns of fluorescently-labeled *E. coli hipA* mutants in the channels of a microfluidic device suggested that slow-growing persister cells were already present in the bacterial culture prior to antibiotic exposure, suggesting that the persistent state may partially stem from stochastic mechanisms.[13] This study led to the identification of two different persister types. Type I persisters are non-growing cells that enter at stationary phase, a dormant state that protects them from the lethal action of several antibiotics known to affect

mainly actively growing cells. Type II persisters do not require a starvation signal to enter the persister state and are continuously generated during exponential growth in a fashion that seems to depend on the population size. Although Type I and II persisters exist in wild-type populations of *E. coli*, their differentiation has only been achieved using time lapse single cell microscopy. Bacterial cells were also shown to still express proteins for a short period of time prior to entering into full dormancy, indicating a gradual downregulation of essential cellular processes during switching to the persistence state.[15]

While HipA contributes to persistence in *E. coli*, the absence or poor conservation of *hipA* in other bacteria that have exhibited persistence suggested the existence of other persistence mechanisms. Several other genetic screens have led to identification of additional metabolic genes that function in cell dormancy, including GlpD[16], an enzyme that functions in glycerol-3-phosphate metabolism, PhoU[17], a negative regulator that inhibits energy metabolism and nutrient transport, the global regulators DksA and DnaKJ[18], and the HipA-like toxin proteins RelE and MazF.[19 - 21] Transient ectopic overexpression of chaperone DnaJ and PmrC were also shown to increase the number of persisters by up to 1000-fold.[19]

There is also increasing evidence that bacterial communication via chemical signaling may play a role in establishing persistence. Recently, indole signaling has been implicated in triggering persistence, leading to enhancement of persister formation in *E. coli* by ~10-fold in response to multiple antibiotics.[22] This indole signaling is dependent on activation of the OxyR and phage-shock pathways and enables the bacterial community to alter its frequency of persistence as a survival mechanism. Another mediator of bacterial cell-cell communications, the quorum-sensing peptide CSP pheromone was also implicated in the formation of stress-induced multidrug-tolerant persisters in the oral pathogen *Streptococcus mutans*, the leading etiological agent of dental biofilm.[23] In addition, gaseous ammonia released by stationary phase bacterial cultures was found to modify the antibiotic resistance spectrum of bacterial neighbors.[24] Ammonia release increases the level of intracellular polyamines, which modulates membrane permeability to different antibiotics.

These results suggest that persisters may form through independent parallel mechanisms and do not follow a single linear regulatory pathway. The underlying commonality is that each of these mechanisms leads to a small subset of quiescent or slowly-dividing cells within an otherwise rapidly dividing population. The fact that the great majority of candidate persister genes have been identified in *E. coli* leaves open the question of whether persistence mechanisms are universal or species-specific. For example, *P. aeruginosa* infection in cystic fibrosis patients is thought to be exacerbated by persister cells in biofilm formation and subsequent recalcitrance to antibiotic treatment.[25] However, the majority the *E. coli* persister gene candidates do not have confirmed homologs in *P. aeruginosa*. An independent screen of a transposon insertion library from *P. aeruginosa* identified a separate list of genes, including a putative DNA helicase and type IV pilus response regulator, as putative persister genes.[26] Another study developed computational algorithms based on systems biology data such as transcriptomics profiles and functional interactions networks to predict novel *M. tuberculosis* genes required for long-term persistence in mouse lungs.[27] In this study, 18 novel genes were

experimentally validated to play a role in persistence. To date, clear understanding of the molecular mechanisms that regulate persister formation has yet to emerge.

Key gene/pathway	Mechanism	Ref
Toxin/antitoxin		
HipA/B	Kinase that phosphorylates EF-Tu	[11-12]
RelE	Ribosome-dependent endonuclease	[20]
RelA	(p)ppGpp synthetase	[35-36]
TisB	Antimicrobial peptide that opens membrane channel	[38-40]
MazF	Endonuclease	[21]
Other genes		
GlpD	Glycerol-3-phosphate metabolism	[16]
PhoU	Negative regulator of energy metabolism	[17]
DksA	Transcriptional regulator of rRNA	[18]
DnaKJ	Chaperone	[18]
PmrC	Transfer of phosphoethanolamine to lipidA	[19]
DinG	DNA helicase in *P. aeruginosa*	[26]
PilH	Type IV pilus response regulator in *P. aeruginosa*	[26]
Other molecules		
Indole	Activation of OxyR and phage-shock pathways	[22]
CSP	Quorom-sensing peptide	[23]
Ammonia gas	Increase in intracellular polyamines	[24]

Table 1. Selected molecular pathways that lead to bacterial persistence

4. Toxin/Anti-toxin (TA) modules

One common gene family that has been linked to bacterial persistence is the TA loci, which function in adaptation to rapidly changing environmental conditions in many bacteria and Archaea.[28] TA modules are present in the genome of diverse bacteria, with more than 50 modules in *Mycobacterium tuberculosis*, a pathogen that enters dormancy as part of its disease lifecycle. Type II TA modules typically encode for a pair of co-transcribed stress-inducible proteins, a stable toxin that inhibits cell growth, and a more labile anti-toxin that regulates the activity of its cognate toxin. (Fig. 3) The toxin and anti-toxin form a tight complex and repress their own expression. Depletion of the anti-toxin leads to release of its cognate toxin, which interferes with an essential cellular target, such as mRNA, DNA gyrase, or DNA helicase, to induce cell cycle arrest or inhibit metabolic functions. There are three types of antitoxins: type I TA antitoxins encode small antisense RNAs that repress toxin gene translation, type II loci

encode protein antitoxins, and type III loci express small RNA antitoxins.[29 - 31] It should be noted that the term 'toxin' can be considered a misnomer, since the toxin genes do not kill the bacteria, but rather repress cell growth.

Since the initial mapping of the *hipA* toxin to a TA locus, several other studies have linked TA function to bacterial persistence. *E. coli* becomes dormant if toxin levels exceed a specific threshold, with the amount determining the length of time bacteria remains in dormancy.[32] Persister cells increased their TA expression levels, and deletion of ten TA loci that encoded for mRNA endonucleases in *E. coli* led to a marked reduction in frequency of persister formation.[33] Furthermore, overexpression of the toxin can induce a persistence state from which cells can be resuscitated by expression of anti-toxin gene transcription.[34] This evidence supports a model in which TA loci play a key role in switching on the persistence state in response to environmental stressors.

Other TA loci, in addition to *hipA/B*, have been implicated in mediating persistence. For example, the toxin RelA has been shown to be required for the long-term survival and persistence of *M. tuberculosis* in mice.[35] Interestingly, RelA encodes for (p)ppGpp synthetase, which functions in the synthesis of (p)ppGpp, or guanosine tetra-and pentaphosphate, a signaling molecule that has been shown to be indicative of the persistent state.[36] (p)ppGpp is a central mediator of the stringent response, which modulates cell expression to survive stress and nutrient limitations.[37] Persisters exhibit relatively higher levels of (p)ppGpp, which is consistent with the slowed growth rate and metabolically inactive state in persisters. Damage of DNA induces the SOS response and expression of the TisB toxin, an endogenous antimicrobial peptide that causes persister formation by opening an ion channel.[38 - 40] This decreases the proton motive force and ATP levels, leading to target shutdown and a dormant, drug-tolerant state.

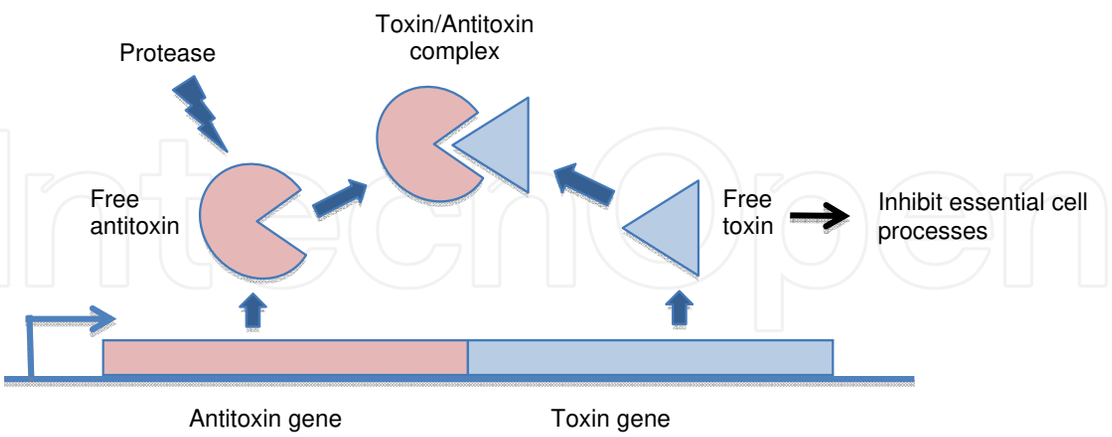

Figure 3. Co-expression and regulation of the TA loci. The anti-toxin regulates toxin activity by forming a tight complex with the toxin. The antitoxin is generally produced at a higher rate than the toxin and is degraded by cellular proteases.

Since both toxin and anti-toxin transcripts are co-expressed from a single promoter, the imbalance between the two transcripts is primarily caused by accumulation of specific

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proteases, such as the Lon protease in *E. coli*, that target the anti-toxin for degradation. Deletion of *lon*, but not other protease-encoding genes, led to a decrease in persistence, indicating that Lon plays in specific role in induction of persistence.[33] Lon was also identified in a screen of a *P. aeruginosa* PAO1 luciferase fusion library as a differentially-regulated gene upon exposure to a sub-inhibitory concentration of gentamicin and was shown to be essential for biofilm formation.[41]

5. Isolation of bulk and single cell persisters

Persisters are a difficult cell population to manipulate, due to their transient nature, low frequency, and mechanistic heterogeneity. A variety of methods have been utilized for persister enrichment and have leveraged *hipA* mutant *E. coli* strains as the starting material to maximize persister numbers. Persister cells have been isolated based on sedimentation of surviving cells following antibiotic treatment.[3, 14] Transcriptomics profiling from the persister population indicated that genes involved in energy production were downregulated, consistent with a dormant phenotype. However, since each antibiotic and microbe exhibits unique killing and survival kinetics, respectively, treatment to isolate persisters is highly dependent on the individual system. Prolonged exposure to antibiotic agents may also activate stress response mechansims, which have been recently linked to induction of persistence. Another isolation method utilized an *E. coli* strain expressing a green fluorescent protein (GFP) reporter fused to a ribosomal RNA promoter *rrnBP1*.[42] Since persisters display low metabolic activty, sorting of dimly green cells by FACS will concentrate persisters that exhibit little of no transcription from the *rrnBP1* promoter. Finally, persisters have also been isolated by using a combination of alkaline and enzymatic lysis that targets the cell membrane and kills normally growing cells.[43] This strategy took 25 min compared to >3 hrs for previous methods and hence is less likely to induce a stable stress response. Furthermore, persisters isolated with this protocol did not exhibit activation of the SOS response, indicating that stress response was not activated.

6. Therapeutic strategies that target bacterial persistence

Initially, investigators sought to identify the genetic determinants that mediate persister formation as potential targets to prevent or reverse persistence. Given the number of disparate genes that appear to be involved in persistence, such an approach may prove to be difficult. Nevertheless, identification of bacterial proteins that are essential even in persisters can provide novel targets for drug development. Since persisters exist in a slowed metabolic state, it is likely that changes in environmental parameters can shift pathogen metabolism from persistence to a replicating state. In the last several years, compounds have been identified that have exhibited promise in the switching of persisters into growing cells susceptible to antibiotic killing or in the direct killing of persisters. (Table 2) These strategies can be integrated with

current antibiotics regimens to develop novel viable therapies for treatment of infectious disease.

6.1. Metabolite stimulation of aminoglycoside-mediated bacterial killing

A promising strategy for the eradication of persistent bacteria is the combination of an antibiotic that kills actively replicating bacteria with a metabolite that may enhance the susceptibility of the persistent bacteria to antibiotics. An elegant example of this strategy was the addition of metabolites to stimulate cellular metabolism and switch *E. coli* and *S. aureus* persisters back to the wild-type state to be susceptible to aminoglycosides.[44] Multiple carbon sources that maximize coverage of glycolysis, the pentose-phosphate pathway and the Entner-Doudoroff pathway were tested for their ability to potentiate aminoglycosides against *E. coli* persisters. Metabolites that enter upper glycolysis, including glucose, mannitol, and fructose, and one that enters lower glycolysis, pyruvate, led to a reduction in persister viability in response to the aminoglycoside gentamicin, by three orders of magnitude. Other metabolites that enter the latter two pathways (e.g. arabinose and ribose) did not have a significant effect on persister death. This potentiation was found to be specific to aminoglycosides, and did not occur in persisters exposed to quinolone or β-lactam antibiotics, which target DNA and the cell wall, respectively. The metabolic stimuli were found to generate a proton-motive force (PMF), which facilitates aminoglycoside uptake and subsequent bacterial killing. Treatment of persisters with an inhibitor of PMF, the ionophore CCCP, abolished aminoglycoside potentiation by the metabolite/gentamicin treatment. These results indicate that persisters, although dormant, are nevertheless primed for metabolic uptake and energy metabolism.

In both an *E. coli* biofilm model and a chronic biofilm-associated infection in mice, a combination of mannitol and gentamicin reduced biofilm viability by several orders of magnitude compared to antibiotic alone. In the infected mice, the dual treatment inhibited bacterial spread to the kidneys, compared to no treatment or antibiotic alone, demonstrating the feasibility for potential clinical use. Metabolite-enabled killing of persisters was also shown to be effective in the Gram-positive pathogen *Staphylococcus aureus*, although with fructose as the most effective metabolite, due to the differential expression of metabolite transporters in *S. aureus*. Thus, delivery of PMF-stimulating metabolites may be a novel strategy to complement current aminoglycoside treatments to generate more effective antibacterial therapies.

6.2. Hyperactivation of ClpP protease kills persisters

Modulation of target protein function that is lethal for microbes is a novel approach for persister cell elimination. In a recent paper, activation of the ClpP protease by the antibiotic acyldepsipeptide 4 (ADEP4) was shown to kill persister cells by degrading over 400 cellular proteins.[45] ADEP exhibits anti-bacterial activity against Gram-positive bacteria *in vitro* and in several rodent models of bacterial infection.[46] ClpP is a proteolytic subunit that normally pairs with different ATPase regulatory subunits to degrade misfolded proteins in the bacterial cytoplasm. Binding of ADEP to ClpP maintains the catalytic chamber of ClpP in an open configuration to enable promiscuous cleavage of proteins and decouple protein degradation from dependence on ATP hydrolysis. Null mutants of *clpP* were resistant to ADEP4, but were

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found to be highly resistant to killing by multiple antibiotics. This uncontrolled proteolysis ultimately resulted in bacterial autolysis and cell death. Treatment with ADEP4 exhibited a decrease in cell count of *S. aureus* stationary cells by 4 $\log_{10}$ in two days. Conventional antibiotics, including rifampicin, linezolid, and ciprofloxacin, were inactive against stationary phase *S. aureus*. However, treatment with a combination of ADEP4 and a conventional antibiotic led to eradication of *S. aureus* persisters in growing and stationary cultures. Furthermore, this combination treatment cleared a chronic biofilm infection in a mouse model that was previously untreatable with just conventional antibiotics.[45] These studies demonstrate that persisters are not invulnerable to killing and that ADEP4 is an effective antibiotic against persisters in a deep-seated biofilm infection. Conventional antibiotics were likely not effective against persisters due to pathogen tolerance rather than the inability to diffuse within the biofilm.

Compound	Mechanism	Ref
Metabolites (e.g. mannitol, fructose)	Metabolite stimulation to generate a proton-motive force for aminoglycoside-mediated killing	[44]
ADEP4	Activation of the Clp protease to promiscuously cleave target proteins in persisters	[45]
HT61	Drug compound depolarizes and breaks down bacterial cell wall	[47]
C10	Drug compound killed persisters in combination with antibiotics	[48]
Pyrazinamide	Byproduct pyrazinoic acid activates multiple essential cellular processes	[53]
Diarlyquinolines (TMC207)	Inhibitor of ATP synthase to block energy metabolism in TB	[55]
Imidazopyridines Benzimidazoles Thiopenes	Drug compounds that reduced ATP content in both replicating and persistent Mycobacteria	[58]
Muropeptides	Activation of PrkC to stimulate translation	[60]
Rpf (Resuscitation promotion factor)	Degradation of peptidoglycan as mediators to induce cell growth	[61]

Table 2. Strategies to defeat bacterial persistence

6.3. Drug screening against metabolically-inactive bacteria

Small molecule library screens have been performed to identify compounds that specifically target persisters. A quinolone-derived library was screened against non-multiplying *S. aureus* using a long-term stationary phase culture.[47] One compound in particular, termed HT61, exhibited 6 logs killing compared to 1 log for commercially-available antibiotics, such as amoxicillin and linezolid, in *S. aureus* persisters and clinical methillicin-resistant *S. aureus* strains. HT61 acts via depolarization of the bacterial cell membrane and subsequent breakdown of the cell wall. HT61 also displayed significant bactericidal effects against other Gram positive pathogens, including *Streptococcus pyogenes* and *Streptococcus agalactiae*, but was less potent for actively-replicating *S. aureus* and not effective against Gram negative bacteria such as *E. coli* and *Klebsiella aerogenes*. In a mouse skin bacterial colonization model, HT61 effectively killed surface *S. aureus* and did not induce adverse effects in a minipig skin model. Importantly, HT61-resistant *S. aureus* were not detected even after 50 passages of exposure, suggesting that compounds that target persisters may bypass development of antibiotic resistance in normally replicating bacteria.

Another screen was performed to identify compounds from a library composed of 6800 chemicals, based on scaffolds and physicochemical properties, that can effectively kill persisters.[48] Compounds were downselected based on enhanced killing of *E. coli* K-12 in combination with both ampicillin and norfloxacin, two antibiotics with different mechanisms of action. In particular, one compound denoted C10, 3-[4-(4-methoxyphenyl)piperazin-1-yl]piperidin-4-yl biphenyl-4-carboxylate, exhibited a marked decrease in *E. coli* and *Pseudomonas aeroginosa* persistence frequency in combination with fluoroquinolone antibiotics, but did not affect normal antibiotic-sensitive cells. While persisters were killed when treated with a combination of C10 and norfloxacin, they also exhibited fast re-growth in the presence of C10 alone, suggesting that C10 may induce reversion of dormant persisters into replicating antibiotic-sensitive cells. It will be necessary to identify the binding target of C10 to more fully understand the mechanism of action on bacterial persistence.

6.4. Combination therapy to treat tuberculosis

Combination treatments that target both replicating and persistent bacteria will likely prove to be an effective strategy to combat chronic infections. A good example of this approach is the multiple drug treatment of tuberculosis (TB), a global disease mediated by *Mycobacterium tuberculosis,* that causes ~2 million deaths per year. Dormant TB can survive within a host for decades, thus requiring long-term treatment. Treatment of active *M. tuberculosis* by a front-line cocktail of four drugs, isozianid, rifampicin, pyrazinamide, and ethambutol, can be effective in controlling the disease in the first several months. Isoniazid is effective against rapidly growing bacilli in lesions, whereas rifampicin and pyrazinamide are thought to be more effective in killing persistent bacilli.[49] These persistent bacilli localize in macrophages within tissue cavities. Upon exposure in a low O_2 (hypoxic) and nutrient environment, they enter a low-metabolic, dormant state by downregulation of protein and nucleic acid synthesis and formation of a thick cell wall. TB persister cells escape both host immune detection and killing by isoniazid, which can lead to disease relapse upon exit out of the dormant state in the host.

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Interestingly, the mechanism of action of the front-line drug pyrazinamide, known for >50 yrs, has only recently been deciphered. Pyrazinamide is activated by a mycobacterial amidase to yield the bioactive compound pyrazinoic acid, which has been linked to multiple essential cellular processes, including ribosome function[50], fatty acid synthase II[51], and respiratory activity.[52] Trapping of pyrazinoic acid in the cell leads to a rise in acidity, which kills the pathogen.[53] Other existing antibiotics, such as moxifloxacin and gatifloxacin, have also been shown to enhance rates of *M. tuberculosis* persister death when combined with conventional drug treatments.[54]

Although in a latent state, *M. tuberculosis* still performs respiratory energy conversion to maintain requisite metabolic functions, providing a potential target for novel drug development. Dormant mycobacteria were found to express a functional ATP synthase to generate energy that drives basal cellular reactions. A newly-discovered inhibitor of ATP synthase, diarylquinoline TMC207, was recently identified as a potent anti-mycobacterial compound against replicating *M. tuberculosis*.[55] Significantly, nanomolar concentrations of TMC207 were able to kill dormant pathogen by blocking ATP synthase activity at a higher efficacy than observed for replicating mycobacteria, despite ATP synthase being transcriptionally downregulated in latent *M. tuberculosis*.[56] TMC207 also exhibited potent sterilization in an infected lung mouse model, comparable to the triple drug combination of rifampicin, isozianid, and pyrazinamide. The high efficacy of TMC207 may stem from killing of both actively-growing and dormant pathogen. These results suggest that basal ATP synthase activity is essential for the survival of dormant *M. tuberculosis,* and ATP homeostasis may represent a viable target for development of persister-directed drugs. TMC207 is presently in Phase II clinical trials for TB treatment. Furthermore, medicinal chemistry optimization efforts led to generation of novel diarylquinoline chemotypes that targeted Gram-positive pathogens, including *S. aureus* in replicating planktonic and metabolically resting biofilm states, potentially broadening the antibacterial spectrum of diarylquinoline-based antibiotics.[57]

To further identify novel inhibitors against dormant mycobacteria, a hypoxic model system was established to screen >600,000 compounds for those that lowered ATP content in a non-replicating *M. bovis* BCG recombinant strain.[58] The screen yielded 140 non-cytotoxic compounds, including imidazopyridines, benzimidazoles, and thiopenes, which modulated respiratory function against both replicating and non-replicating mycobacteria. Reduction of cellular ATP levels was shown to correlate with cell death in bacteria treated with the three compound clusters. Thus, these clusters may form the basis of antibiotic compound development against mycobacteria.

6.5. Bacterial factors that trigger exit from dormancy

Sporulation is another form of persistence in which both pathogen and environmental microbes enter a metabolically-inactive state and become resistant spores under unfavorable conditions. For example, in nutrient-poor environments, *Bacillus* and *Clostridium* species undergo tightly regulated transcriptional and morphological changes to form a dormant but robust spore that is resistant to extreme stress conditions, such as high temperatures, desiccation, and toxicity. In the laboratory, *Bacillus subtilis* spores have been shown to germinate and

exit the spore state in response to treatment with various nutrients, such as amino acids.[59] Binding of these nutrients to receptors on the inner membrane of the spore leads to rehydration of the cell interior and breakdown of the peptidoglycan spore layer. *B. subtilis* has also been shown to exit dormancy in response to peptidoglycan-derived muropeptides, consisting of a disaccharide-tripeptide with a meso-diaminopimelic acid (m-DAP) residue in the third position.[60] Peptidoglycan from growing cells, but not stationary cells, have been shown to more effectively induce germination, suggesting that these muropeptides may function as signaling molecules that can stimulate dormant cells. In response to muropeptide binding to its extracellular domain, the serine/threonine kinase PrkC has been shown to phosphorylate elongation factor G (EF-G), which modulates ribosomal activity and initiation of translation to induce microbial exit from dormancy.[60]

Other protein factors expressed by microbes have been reported to stimulate growth of dormant cells. The environmental microbe, *Micrococcus luteus*, secretes a resuscitation-promoting factor (Rpf), which can stimulate exit from dormancy when added to *M. luteus* cultures.[61] *M. luteus* Rpf has been shown to hydrolyze peptidoglycan at picomolar concentrations and stimulate aged cultures of *M. tuberculosis*, thus exhibiting cross-species activity.[62, 63] *M. tuberculosis* expresses five endogenous Rpfs that function in reactivation of chronic tuberculosis in animal models.[64] Although the exact function of Rpfs remains unclear, it has been suggested that Rpf-mediated proteolysis of peptidoglycan can generate muropeptides for PrkC-like kinases to activate protein translation.[65]

7. Conclusion

The rise in antimicrobial drug resistance, alongside the failure of conventional research efforts to discover new antibiotics, will eventually lead to a public health crisis that can drastically curtail our ability to combat infectious disease. Bacterial persistence is an underexplored mechanism by which to develop novel treatments to complement or extend the current repertoire of antibiotics.[66 - 68] Although persisters do not cause overt disease, they act as a pool from which bacteria can emerge from dormancy to cause recurrent infection. Mechanisms of persister formation appear to be highly redundant across different bacterial species, which contributes to the difficulty in identification of universal mechanisms to target and eradicate persistence. To date, the more successful strategies in the lab have been to target cell functions, such as basal energy metabolism and cell wall integrity, that are also essential for persister cell maintenance.[69] Of particular note, addition of metabolites such as mannitol or fructose was shown to potentiate aminoglycoside-mediated killing by generating a proton motive force to stimulate aminoglycoside uptake.[44] Several medicinal chemistry strategies to screen for small molecules effective against persisters have also identified lead targets for potential optimization and rational drug design.

By developing treatments against both persisters and replicating pathogens, it may be possible to shorten antibiotic regimens, especially for deep-seated diseases such as tuberculosis, and reduce relapse rates in patients. Another advantage to combination therapies is potential

extension of the useful life of current antibiotics to kill pathogen at a faster rate, and thus slow down the further emergence of antibiotic resistance. Additional strategies to optimize pulse-dosing regimens using multiple antibiotics that include anti-persister drugs may be able to sterilize particularly recalcitrant chronic infections. These types of therapies may be designed for the individual patient as part of an increasingly personalized approach to medicine. Aside from the clinical relevance of bacterial persisters, non-genetic heterogeneity has been found to play important roles in other systems, including susceptibility of cancer cells to treatment[70], host response to viral infection[71], and bacterial responses to other stresses.[72] Thus, understanding the mechanisms of cell-to-cell variability will provide insights into the general adaptation of life to variable environments.

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