ACS OMEGA

Perspective

Cite This: ACS Omega 2017, 2, 7400-7409

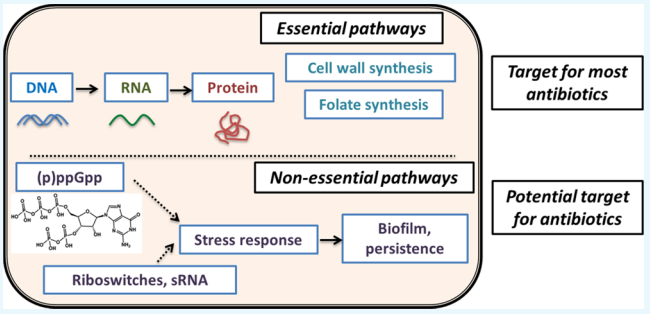
http://pubs.acs.org/journal/acsodf

Antibiotic Resistance: Current Perspectives

Anushya Petchiappan and Dipankar Chatterji*

Molecular Biophysics Unit, Indian Institute of Science, Bangalore 560012, India

ABSTRACT: Antibiotic resistance is one of the most serious challenges that the world is currently facing. The number of people succumbing to drug-resistant infections is increasing every day, but the rate of drug discovery has failed to match the requisite demands. Most of the currently known antibiotics target the three essential pathways of central dogma. However, bacteria have evolved multiple mechanisms to survive these antibiotics. Consequently, there is an urgent necessity to target auxiliary pathways for the discovery of new drugs. Metabolism-related and stress-associated pathways are ideal in this regard. The stringent response pathway regulated by the signaling nucleotides (p)ppGpp is an attractive target as inhibition of the pathway would in turn decrease the persistence and long-term survival of pathogenic bacteria. In this perspective, we focus on the recent design of small molecule analogues of (p)ppGpp that have yielded promising results in terms of growth and biofilm inhibition. Additionally, we discuss how targeting small RNAs and riboswitches, as well as antimicrobial peptides, would help combat drug-resistant infections in the near future.



1. INTRODUCTION

Antibiotics have been the foremost weapon wielded by mankind in our war against pathogenic microorganisms, but several decades after the discovery of antibiotics, the microbes have fought back valiantly with a host of strategies to survive them. Prior to the discovery of antibiotics, a microbial infection was most often synonymous with death of the infected human being. Sulfa drugs were the first tools used to fight pathogens.[1] Subsequently, the serendipitous discovery of penicillin in 1929 heralded the "golden era of antibiotics", in which several new antibiotics were discovered by humans that helped them win the war against these microbes.[1,2,3] Since then, antibiotic resistance has become a global health concern. More than 2 million people every year are infected with antibiotic-resistant infections in the United States alone. Drug-resistant infections lead to approximately 23 000 deaths in the United States and 25 000 deaths in Europe per annum, and the number is much higher in developing countries.[4] Apart from the loss of lives, these infections also lead to billions being spent on healthcare and treatment. The current pace of drug development is insufficient to mitigate the severe threat of drug-resistant infections. This necessitates an urgent need to develop a deeper understanding of the reasons behind the emergence of antibiotic resistance and to develop tools to combat it.

1.1. Emergence of Antibiotic Resistance. The word "antibiotic" is derived from "anti" (meaning against) and "biotikus" (meaning fit for life), and therefore, it literally means "life-killing". The term antibiotic has been hereafter used in this review to describe both naturally derived and chemically synthesized antibacterial drugs. After the clinical success of penicillin, the microbial world was explored for the presence of other similar antibiotics. Several metabolites extracted from environmental microbes were studied for their effect on growth of pathogens. This mining for antibiotics from the environment proved extremely successful, leading to the discovery of several potent antibiotics like streptomycin.[3] Subsequently, medicinal chemistry helped the synthesis of several chemically derived drugs like *para*-aminosalicylic acid. These were synthesized based on natural antibiotic scaffolds, and they helped to expand the spectrum of antibiotics available. The naturally derived antibiotics evolved to cross bacterial cell membranes, but chemically synthesized drugs often needed several modifications to reach the same efficacy. Most antibiotics target the cellular processes of translation, transcription, replication, and cell wall synthesis (Figure 1).[3] Antibiotics often exert complex effects in vivo and may have multiple cellular targets. The most commonly used antibiotics include the β-lactams, which inhibit cell wall synthesis and include cephalosporins, carbapenems, and penicillins. Other cell wall synthesis inhibitors include glycopeptide antibiotics like vancomycin. Protein synthesis inhibitors include aminoglycosides (e.g., kanamycin), tetracyclines, chloramphenicols, macrolides (e.g., eyrthromycin), oxazolidinones (e.g., linezolid), and streptogramins (e.g., pristinamycin). Rifamycins constitute transcription inhibitors, and quinolones (e.g., ciprofloxacin) inhibit DNA synthesis by binding to gyrase. Another class of antibiotics commonly used are the sulfonamides, which are inhibitors of folate synthesis.

The World Health Organization (WHO) has listed several pathogens that have developed high levels of resistance across the world.[1,3] Among these, the "ESKAPE" pathogens are

Received: September 14, 2017
Accepted: October 11, 2017
Published: October 30, 2017

DOI: 10.1021/acsomega.7b01368
ACS Omega 2017, 2, 7400−7409

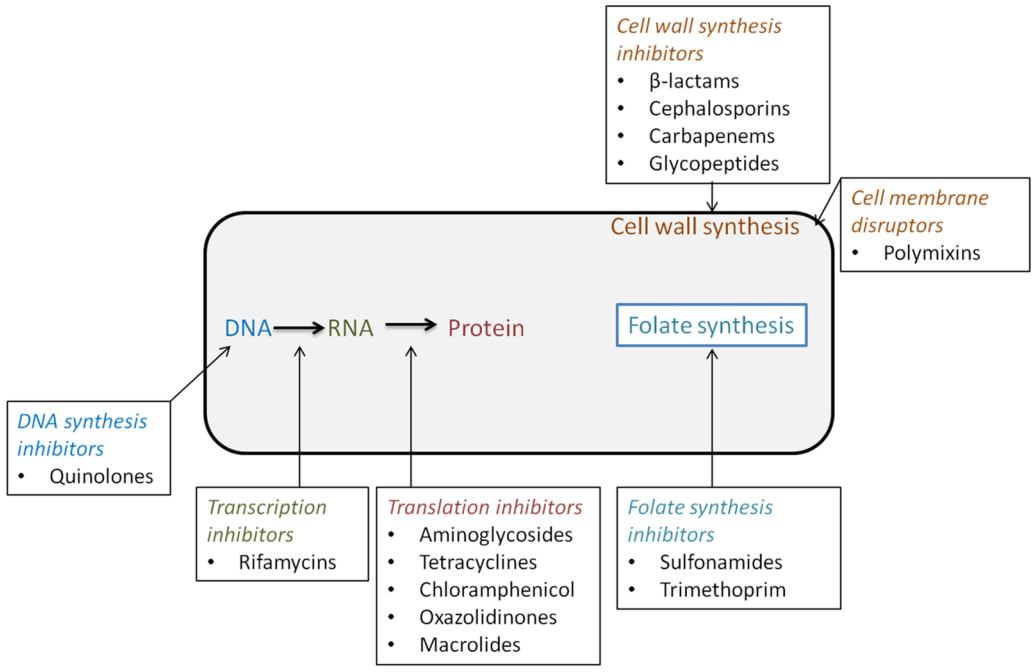


Figure 1. Different classes of antibiotics and their mechanism of action.

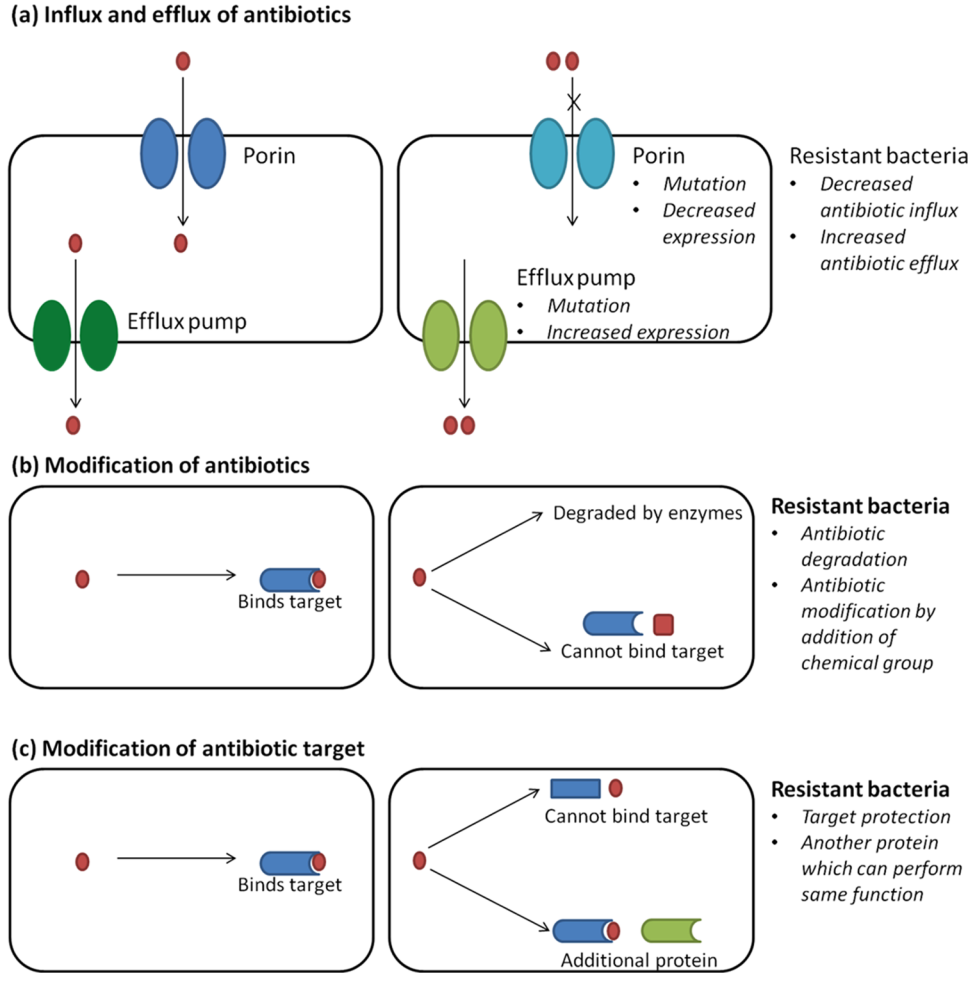


Figure 2. Mechanisms of antibiotic resistance. There are three main ways by which bacteria can increase their resistance: (a) varying the efflux of antibiotics; (b) modifying the antibiotics; or (c) modifying their targets.

DOI: 10.1021/acsomega.7b01368
ACS Omega 2017, 2, 7400−7409

extremely important and include *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.[5] *Enterobacteriaceae* spp. (*Escherichia coli*, *K. pneumoniae*, *Enterobacter* spp., *Serratia* spp., *Proteus* spp., *Providencia* spp.), *Helicobacter pylori*, *Salmonella* spp., *Neisseria gonorrhoeae*, *Streptococcus pneumoniae*, *Haemophilus influenza*, and *Shigella* spp. strains have shown high levels of drug resistance. There is growing resistance to carbapenems, cephalosporins, fluoroquinolones, and aminoglycosides. Most *Enterobacteriaceae* strains have developed resistance against third generation carbapenems. *Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB), is the most common reason behind death from an infectious pathogen. Tuberculosis (TB) killed approximately 1.5 million people in 2015, and the emergence of multiple-drug-resistant TB and extensively drug-resistant TB strains has made global eradication of TB an extremely challenging task.[6]

1.2. Mechanisms of Antibiotic Resistance. Understanding the mechanism behind antibiotic resistance is the primary step in developing ways to combat it. Bacteria can be intrinsically resistant to antibiotics or can acquire the trait.[4] For example, *M. tuberculosis* is intrinsically resistant to the β-lactam group of antibiotics as it encodes a β-lactamase enzyme in its genome.[7] Also, most antibiotics have different efficacies in Gram-negative and Gram-positive bacteria due to intrinsic differences in the cell wall composition. Several studies in the past few years have led to the identification of multiple genes responsible for intrinsic antibiotic resistance in bacteria.

Antibiotic resistance can be acquired by bacteria through chromosomal mutations or via horizontal gene transfer from other bacteria. Antibiotic resistance in bacteria can develop mainly due to three mechanisms (Figure 2):

(i) Decreased influx or increased efflux of antibiotics:
Antibiotic entry into the cell is mainly through porins present in the outer membrane.[4] A decreased cellular expression of porins or mutations in the porin genes leads to reduced entry of the antibiotic into the cell, making it less effective. Bacteria possess multidrug efflux pumps that are responsible for the active export of antibiotics from the cell. Several of these pumps have been identified in bacteria, and mutations in their gene sequences or their overexpression leads to increased antibiotic efflux from the cell.

(ii) Modification of antibiotics:
Once the antibiotic enters the cell, the resistant bacteria either enzymatically degrade it or modify it in such a way that it cannot bind its own target.[4] Enzymes that degrade antibiotics are present in several bacteria. Variants of β-lactamases are encoded in bacteria, each of which can degrade several β-lactam antibiotics. Bacteria resistant to all β-lactam antibiotics have emerged, and new classes of β-lactamases have been identified in pathogenic bacteria like *K. pneumoniae*, *E. coli*, *P. aeruginosa*, etc. Also, there are several known enzymes that can transfer chemical groups like phosphate, acyl, nucleotidyl, etc. to the antibiotic molecules. These modified antibiotics then have decreased affinity to their targets, thereby reducing their efficacy. Aminoglycoside antibiotics are known to be modified by acetyltransferases, nucleotidyltransferases, as well as phosphotransferases.

(iii) Modification of the antibiotic target: Another mechanism of decreasing antibiotic efficacy is to mutate, modify, or protect their cellular targets thereby interfering with the binding of the antibiotics.[4] However, only those mutations that lead to reduced antibiotic binding without affecting the protein activity are favored. Rifampicin-resistant *M. tuberculosis* strains, for example, contain mutations in the *rpoB* gene encoding the β-subunit of RNA polymerase (target of rifampicin).[7] Similarly, methylation of ribosome confers resistance to ribosome-targeting antibiotics. Methylation of 23S ribosomal RNA (rRNA) by Erm methyltransferase confers resistance to macrolide antibiotics.[4] Bacteria can also acquire a gene encoding a homologue of a drug target that does not bind to the drug. Methicillin-resistant *S. aureus* (MRSA) possesses a penicillin-binding protein PBP2a in addition to the chromosomally encoded PBP.[8] PBP is inhibited by β-lactam antibiotics but PBP2a is resistant to the action of β-lactam antibiotics so the cell can still carry out cell wall synthesis. Alternatively, bacteria can synthesize a molecule mimicking the antibiotic target that binds to the antibiotic and reduces its effective concentration. One such example is the MfpA protein in *M. tuberculosis*, which consists of pentapeptide repeats and mimics the shape and charge of B-DNA. It provides resistance to quinolones by binding to DNA gyrase (target of quinolones) in place of DNA, which reduces the availability of gyrase for binding to quinolones.[9]

Antibiotic resistance can be governed by a network of regulatory proteins, which include sigma factors and transcription factors. For example, in Mycobacteria, the transcription factor WhiB7 is induced upon antibiotic stress and regulates expression of several antibiotic resistance-associated genes like *tap* (a multidrug transporter) and *ermMT* (ribosomal methyltransferase).[10] As a consequence, upregulation of expression of such transcription factors leads to increased drug resistance. Expression of several efflux pumps is also regulated at the transcriptional level.[4]

The genes responsible for antibiotic resistance can be transmitted from one bacteria to another. Because antibiotics are naturally produced by bacteria, the other bacteria in the environment often encode genes that provide resistance to those antibiotics, thereby ensuring their own survival. Such genes form a part of the soil "resistome" and can be transferred to the pathogenic bacteria.[4]

1.2.1. Bacterial Persistence. Another phenomenon that leads to low clearance of bacterial infections is persistence.[11] The killing phase of bacteria by antibiotics has two phases: a rapid killing phase in which most bacteria are killed by the antibiotic and another stagnant phase in which a low number of bacteria persist and are not killed. If the antibiotic is removed, these persister bacteria (or "persisters") again start multiplying within the host. This leads to slow clearance of the infection and recurrent bacterial infections. Persistent bacteria are different from resistant bacteria. The minimum inhibitory concentrations required for inhibiting the growth of persistent bacterial cells is the same as that of the wild type cells, and they are not genetically different. Unlike resistant bacteria, persisters can resume growth only once the antibiotic stress is removed (Figure 3). Persistence is a widespread phenomenon observed in most bacteria. *M. tuberculosis* is one of the main human

DOI: 10.1021/acsomega.7b01368
ACS Omega 2017, 2, 7400−7409

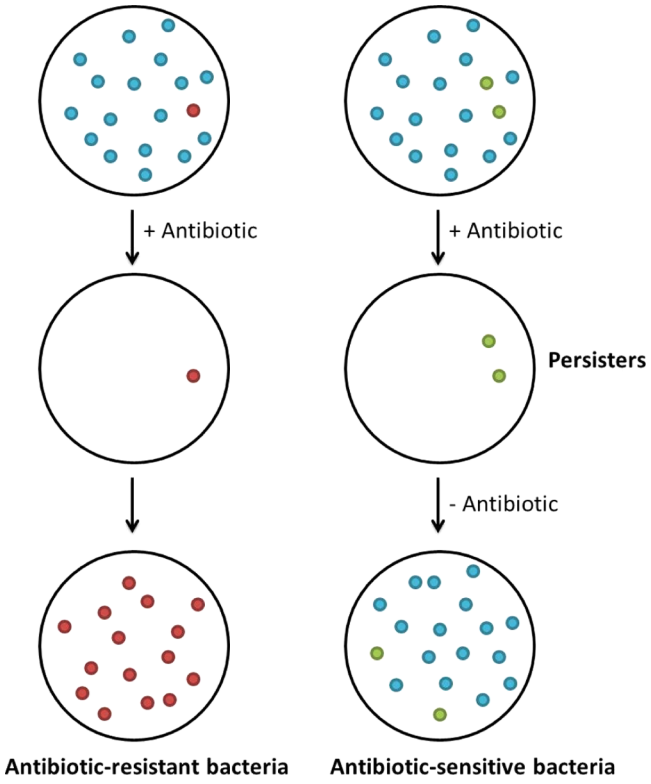


Figure 3. Difference between antibiotic-resistant bacteria and persisters. Resistant bacteria can survive and multiply even in high antibiotic concentration. An isogenic bacterial culture has a low percentage of persisters that survive the antibiotic stress, then regrow once the antibiotic stress is removed. These persisters are as sensitive to antibiotics as the initial population.

pathogens in which persistence has been observed. The treatment for *M. tuberculosis* includes a combination of drugs like rifampicin, isoniazid, pyrazinamide, and ethambutol.[6] The duration of the treatment is a minimum of 6 months and a relapse of infection is commonly observed. Therefore, the clinical burden due to bacterial persistence is a major concern. In this regard, the study of persistence and its underlying mechanisms has become highly significant.

Persisters are often present as a small fraction of cells in an isogenic bacterial culture.[12] This "bet-hedging strategy" is said to be a survival strategy under fluctuating environment conditions. Persistence can be stochastically activated or can be environmentally triggered by stress. The signaling nucleotides guanosine tetraphosphate and guanosine pentaphosphate (collectively referred to as (p)ppGpp) are the key regulators of the stress response in bacteria. Under nutrient-limiting conditions or stress, (p)ppGpp orchestrates a stress response known as "stringent response" in which the entire cellular machinery is reprogrammed to switch from energy-consuming processes (like replication and rRNA synthesis) to those processes essential for survival. The connection between (p)ppGpp and persistence has been well-characterized and (p)ppGpp has been implicated in several pathways leading to persistence.

(p)ppGpp has been proposed to affect antibiotic tolerance of bacteria via an active stringent response or a passive growth arrest. An active stringent response rather than a passive growth arrest has been shown to lead to antibiotic resistance in *P. aeruginosa*.[13] One of the mechanisms by which antibiotics are proposed to kill bacterial cells is by increasing the reactive oxygen species. (p)ppGpp has been shown to improve antioxidant defenses by upregulating specific genes that help in survival during antibiotic stress. Growth arrest leads to inactivity of antibiotic targets thereby leading to survival of bacteria. In a slow growing cell or a dormant cell, processes like replication, translation, and cell wall synthesis are switched off so that the antibiotics cannot act on their targets. Strains devoid of (p)ppGpp have been shown to have lower levels of persistence in cases like *M. tuberculosis*.

Persistence can arise via multiple pathways.[12] Toxin–antitoxin modules (TA modules) are composed of a stable toxin protein that inhibits cell growth and an unstable antitoxin that neutralizes the function of the toxins. Antitoxins can either be proteins or antisense RNA, and are classified based on the mechanism employed by them to counteract the action of the toxins. TA systems have been categorized into different clases based on their mode of action, and the transcriptome of persister cells revealed their upregulation in *E. coli* and *M. tuberculosis*. A model proposing the role of (p)ppGpp involves inhibition of the enzyme exopolyphosphase Ppx by (p)ppGpp, which leads to inhibition in polyphosphate degradation.[14]

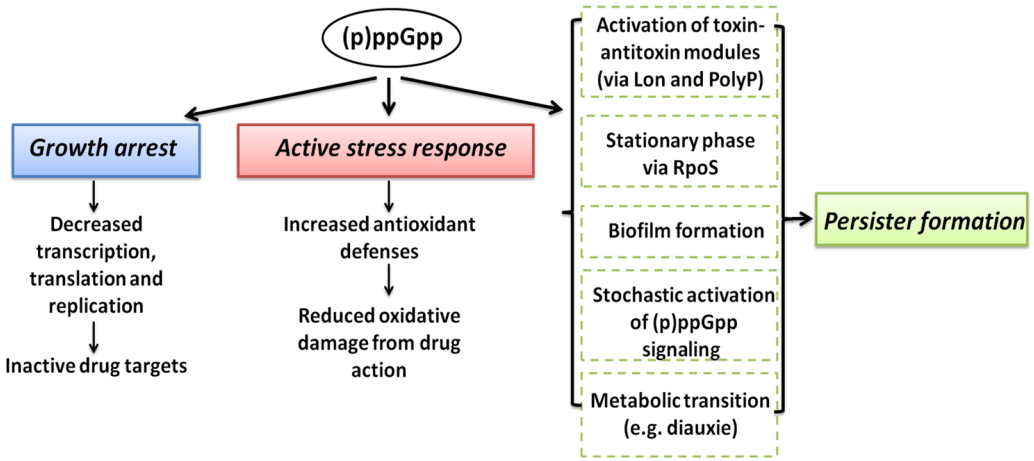


Figure 4. Effect of (p)ppGpp on antibiotic tolerance and persister formation. (p)ppGpp can lead to antibiotic tolerance via passive growth arrest or an active starvation response. It also leads to increased persister formation via multiple mechanisms.

DOI: 10.1021/acsomega.7b01368
ACS Omega 2017, 2, 7400–7409

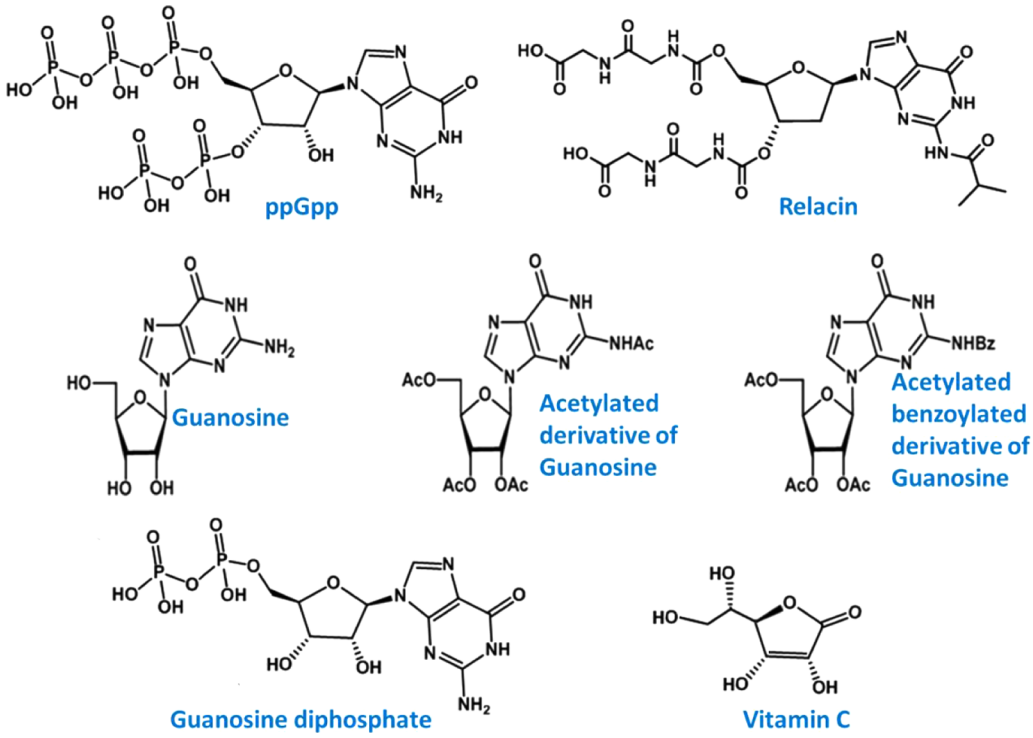


Figure 5. Design of stringent response inhibitors. Relacin is a structural analogue of ppGpp. Guanosine was chemically modified to synthesize acetylated and acetylated benzoylated derivatives that inhibit Rel. Vitamin C is a natural analogue that resembles GDP in structure.

Polyphosphate activates Lon protease, which degrades the antitoxin leading to the toxin level becoming high and causing growth arrest. A bacterial SOS response to DNA damage also triggers persistence via TA-dependent modules or other pathways. *E. coli* lacking SOS genes (e.g., *recA*) display a low frequency of persister formation and are more susceptible to quinolones antibiotics. The stationary phase in bacteria also increases persister formation. (p)ppGpp helps to elevate the stationary phase sigma factor σ^S, which increases persister formation due its role in stress-related pathways.[12] Environmental cues (like stress in phagocytic vacuoles) can trigger persister formation via (p)ppGpp. Persister formation can also be enhanced by treatment with sublethal concentrations of antibiotic. Metabolic transitions during diauxic growth in bacteria have also been shown to increase persister formation.[15] Stress response and persistence have been demonstrated to be linked with increased mutation frequency, which could lead to development of antibiotic resistance (Figure 4).

1.2.2. Biofilm Formation. Biofilms are structured communities of microbes growing on surfaces or at air−liquid interfaces.[16] They are formed when microbes are embedded in a self-produced extracellular matrix composed of proteins, extracellular DNA, lipids, polysaccharides, and water. Several bacterial species like *P. aeruginosa*, *K. pneumoniae*, *S. aureus*, *M. tuberculosis*, etc. have been shown to form biofilms. Biofilms are recalcitrant to antibiotics and the presence of a high number of persisters in biofilms is attributed as the major reason. They have been frequently observed growing on implanted medical devices inside the host. Because these biofilms are highly resistant to most antibiotics, the infections are difficult to eradicate. Anti-biofilm drugs are, therefore, the need of the hour. (p)ppGpp has been implicated in biofilm formation in multiple species like *P. aeruginosa*, *M. tuberculosis*, etc.[16,17]

2. COMBATING ANTIBIOTIC RESISTANCE

The golden era of antibiotics led to the discovery of several important antibiotics, but the last decade has yielded very few potential drugs on account of various reasons. This has set the alarm bells ringing as we may be soon out of antibiotics to treat these drug-resistant infections. Most antibiotics used currently are derived from species like Actinomycetes.[3] In the past, antibiotics were discovered in a randomized manner by mining environmental microbes for their metabolites and testing them against pathogens. Any compound that showed antibacterial activity was considered a putative drug, with no need to decipher its underlying mechanism of action. However, with the advent of genome sequencing, a more targeted approach can be followed for the discovery of new drugs. Transposon mutagenesis led to the generation of libraries with mutants of each gene in an organism. The growth profiles of these mutants or knockout strains reveal information about the essentiality of a gene product, which in turn is the determining factor in deciding its potential as a drug target. Essential genes are considered ideal candidates as any drug targeting the gene product would then cause cell death. This led to the "essential gene paradox" in drug discovery in which only essential genes were considered as drug targets. However, the importance of "conditionally essential" genes has only recently been explored. It is known that pathogens have to survive hostile conditions inside the host. Hence, they have evolved multiple pathways that are triggered only under exposure to specific stress conditions. A particular protein may be, therefore, crucial for their survival inside the host, but may not be needed for growth in vitro. Under normal testing conditions, the mutant strains are grown on rich media and tested for any growth defects on addition of potential antibiotics. A conditionally essential gene, like a starvation-activated gene for example, may not show a phenotype under such medium conditions but would show

DOI: 10.1021/acsomega.7b01368
ACS Omega 2017, 2, 7400−7409

growth defects when the bacteria are grown under nutrient-limiting conditions. Such genes would also make suitable drug targets and in turn expand the available pool of drug targets several fold.

An important parameter for a successful drug is the ability to kill the infecting organism without harming the host. Thus, the macromolecular synthesis pathways of replication, transcription, and translation are often targeted. However, on the flip side, the need for survival drives the pathogens to mutate in such a way that core metabolism remains intact. Thus, it is increasingly being felt that auxiliary pathways need to be targeted where the pressure of mutations will be far less.

With the aim of decreasing bacterial persistence, drugs targeting pathways of persister formation or biofilm formation would be a landmark discovery. These drugs can then be given in combination with the usual drug regimen for a bacterial infection.

3. DEVELOPMENT OF STRINGENT RESPONSE INHIBITORS

As mentioned earlier, the stringent response pathway governed by the signaling nucleotides (p)ppGpp plays a vital role in the stress response of a cell. (p)ppGpp not only affects persistence and biofilm formation but also regulates transcription of various genes required for virulence and long-term survival.[18] As stress conditions (e.g., nutrient limitation) are often encountered by bacteria inside the host, a stringent response is activated. This makes (p)ppGpp an ideal drug target. The absence of a similar pathway in humans is an added advantage. A precise balance of (p)ppGpp levels is required for proper cell function, as too little (p)ppGpp affects survival under stress and too much (p)ppGpp is toxic to the cell due to its effect on GTP levels. One way to affect (p)ppGpp-mediated pathways is to synthesize structural analogues of (p)ppGpp. The concept of inhibitor design deals with the synthesis of substrate or product analogues that can bind to and then turn off key enzymes in the cell. Good expertize in chemistry is essential for this process.

(p)ppGpp is synthesized in vivo by the protein Rel.[18] The first (p)ppGpp analogue designed and synthesized by Wexselblatt et al. was based on a crystal structure of Rel from *Streptococcus equisimilis*, and was named relacin (Figure 5).[19] Relacin is a 2′-deoxyguanosine-based analogue of ppGpp, and glycine−glycine dipeptides are linked to the sugar ring by a carbamate bridge replacing the pyrophosphate residues in ppGpp. In silico analyses revealed that it interacts with Rel through hydrophobic and hydrophilic interactions. Relacin affected sporulation as well as long-term survival in *Bacillus subtilis*. This paved the way for development of other stringent response inhibitors. Subsequently, the same group synthesized relacin analogs with different substitutions at the 3′ and 5′ positions of 2′-deoxyguanosine.[20] One of the analogues was found to be more potent than relacin and showed 80% inhibition of Rel-mediated (p)ppGpp synthesis at 1 mM concentration. The isobutyryl group at the second position in the guanine moiety of relacin was found to be important for its activity. With the aim of synthesizing more potent Rel inhibitors, Syal et al. modified guanosine at this C-2 position to synthesize acetylated and acetylated benzoylated derivatives of guanosine (Figure 5).[21] The acetylated benzoylated compound showed 50% inhibition at 40 μM and 75% inhibition at 100 μM. Addition of this compound led to inhibition of biofilm formation by *M. tuberculosis*. It also led to decreased long-term survival and cellular (p)ppGpp levels in *Mycobacterium smegmatis*. The compound was shown to be cell wall permeable in human lung epithelial cells and nontoxic to H460 cells. With information from the crystal structures of (p)ppGpp bound to its synthetases and effector proteins, more such analogues can be designed and synthesized chemically.

A recent study showed that vitamin C inhibits *M. tuberculosis* growth at high concentrations.[22] Syal et al. proposed vitamin C to be an inhibitor of stringent response and demonstrated its binding to Rel (Figure 5).[23] Addition of vitamin C inhibited (p)ppGpp synthesis by Rel in vitro as well as reduced in vivo (p)ppGpp levels and biofilm formation in *M. smegmatis*. Thus, this opens up opportunities to test vitamin C alone against bacterial infections.

It must be noted that multiple Gram-positive species like *B. subtilis* possess additional (p)ppGpp synthetases that are smaller in size, as compared to Rel, and are upregulated under different stresses. Relacin was found to be ineffective against one such synthetase from *Enterococcus faecalis*.[24] Interestingly, recent studies have shown that some of these small (p)ppGpp synthetases are regulated both by (p)ppGpp as well as RNA.[25] As these synthetases also affect antibiotic tolerance in bacteria, future studies should take them into consideration when designing stringent response inhibitors.

Apart from (p)ppGpp, bacteria employ other nucleotide derivatives like cyclic-AMP, cyclic-di-GMP, cyclic-di-AMP, and cyclic-GMP-AMP as signaling molecules.[26] Each signaling molecule is activated under particular stress conditions; it then activates a cascade of downstream pathways that lead to a particular stress-associated phenotype like biofilm formation. These signaling molecules, similar to (p)ppGpp, regulate several important pathways related to cell division, quorum sensing, virulence, biofilm formation, motility, antibiotic resistance, etc. Disrupting these signaling pathways would therefore affect the bacterial survival, and in the future, more research should be undertaken to develop inhibitors of these signaling nucleotides. This could be achieved by synthesizing structural analogues of these compounds or by targeting their effectors, which can be either proteins or RNA.

4. ANTI-BIOFILM PEPTIDES

As mentioned previously, biofilms are the product of an adaptation mechanism to stress, and eradicating them would help significantly in combating resistant bacterial infections.[27] One class of inhibitors being screened for use against biofilms are peptides.[28] In general, natural antimicrobial peptides are 12−50 amino acids in length. They are cationic, amphipathic, consist of around 50% hydrophobic residues, and vary significantly in sequence and structure. The human cathelicidin peptide LL-37 was demonstrated to have an anti-biofilm effect in *Pseudomonas*.[29] LL-37 is also capable of modulating human immune response and is active against MRSA. Subsequently, chemically synthesized peptides of smaller length have been tested for their anti-biofilm properties. These were mostly designed based on mutation of the natural peptides and then screened for inhibition of biofilm formation and biofilm disruption. Peptides IDR 1002 and HH2 are 12-mers that showed anti-biofilm activity against *S. aureus* biofilms and were subsequently improved to generate peptide 2009 with an overall improved profile.[30] In a subsequent study, D-enantiomeric, including retro (D-amino acid sequence) and retro-inverse (reversed D-amino acid sequence), anti-biofilm peptide 1018 was designed and tested against biofilms of pathogenic bacteria.[31] The peptides were made by utilizing only

DOI: 10.1021/acsomega.7b01368
ACS Omega 2017, 2, 7400−7409

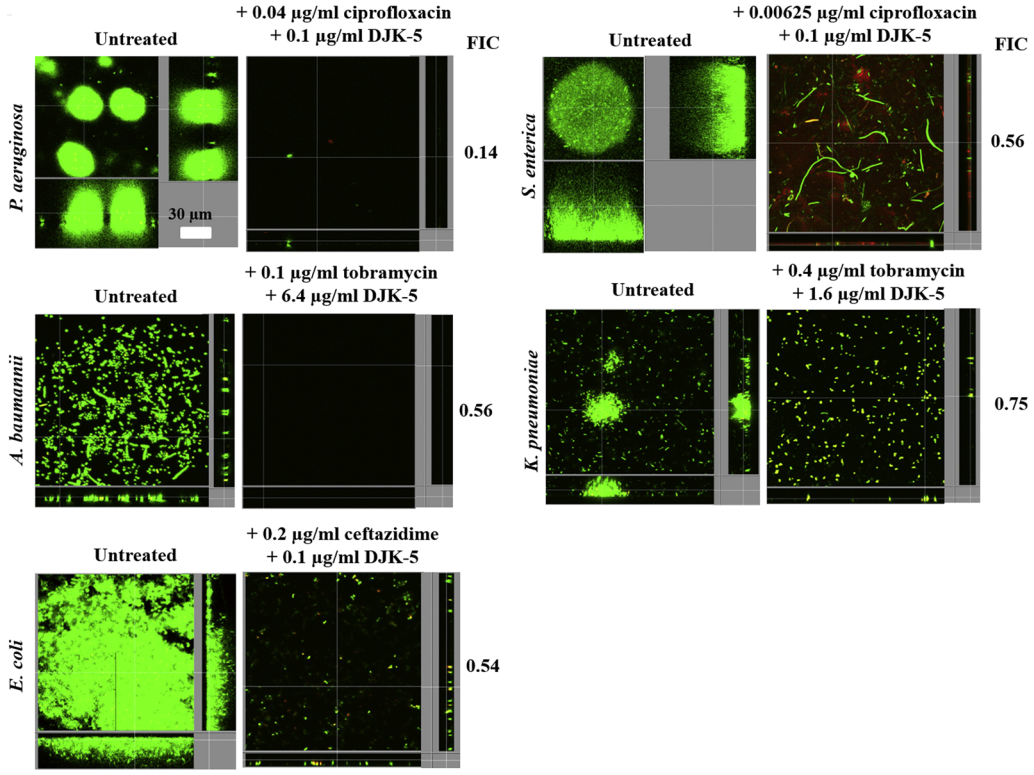


Figure 6. Inhibition of biofilm formation by the D-enantiomer peptide DJK-5 in flow cells. The peptide DJK-5 was provided along with other conventional antibiotics and succeeded in preventing biofilm formation by different pathogenic bacteria. At day 0, a subinhibitory concentration of DJK-5 plus antibiotic was added into the flow-through medium of the flow cell apparatus. The biofilm formation was monitored for 3 days. Subsequently, the live bacterial cells were stained with Syto-9 stain and the dead bacterial cells were stained with propidium iodide (merge shows as yellow to red) prior to confocal imaging. Each panel shows reconstructions from the top in the large panel and sides in the right and bottom panels (*xy*, *yz*, and *xz* dimensions). (Reproduced with permission—de la Fuente-Nunez et al., 2015.)

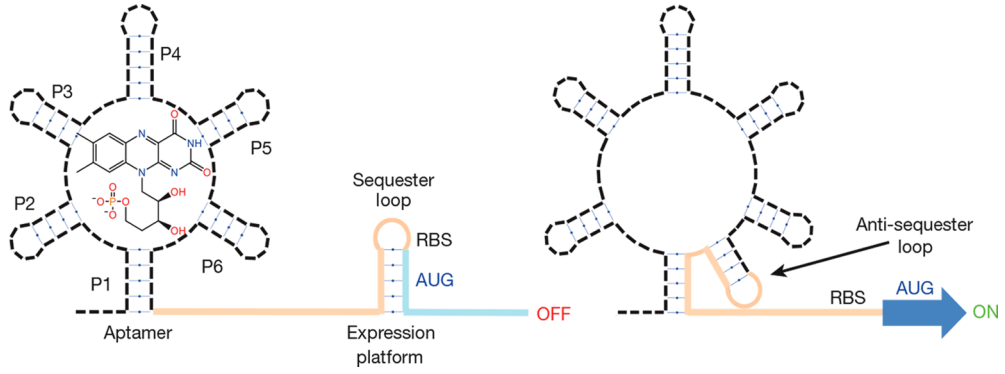


Figure 7. Representation of FMN riboswitch. Upon binding of FMN to the aptamer, there is a conformational change in the expression platform leading to the formation of a terminator/sequester loop, thereby abolishing gene expression. Absence of FMN leads to an alternate conformation involving an anti-terminator/anti-sequester loop that facilitates transcription. (Reproduced with permission: Howe et al., 2015.)

9 of 20 amino acids, and they had 4 charged residues and 7 or 8 hydrophobic residues. These peptides were successful in eradicating mature biofilms and were synergistic with conventional antibiotics. The D-enantiomeric peptides DJK-5 (VQWRAIRVRVIR) and DJK-6 (VQWRRIRVWVIR) showed better activity as compared to that of their L-enantiomeric counterparts as they were protease-resistant (Figure 6). Most of these anti-biofilm peptides are broad-spectrum and inhibit biofilm formation in multiple bacterial species. Some of these anti-biofilm peptides were proposed to target (p)ppGpp but this was later disputed.[32] Other naturally derived peptides and their synthetic derivatives have recently shown promising results in combating mycobacterial infections.[33] As chemical peptide synthesis has become more rapid and economical, peptides with increased efficacy and cellular permeability can be designed. Peptide sequences can be rationally designed such that they specifically target key proteins crucial for virulence and persistence.

5. SMALL RNAS AND RIBOSWITCHES

Protein translation, which requires ribosomal proteins and ribosomal RNA (rRNA), is one of the pathways targeted by most antibiotics. However, other noncoding RNAs have so far failed to be the target of any antibiotic available in the market.

DOI: 10.1021/acsomega.7b01368
ACS Omega 2017, 2, 7400−7409

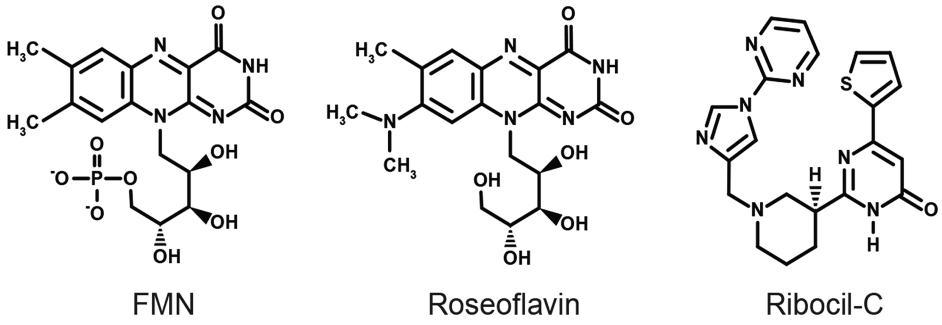


Figure 8. Structure of FMN, roseoflavin antibiotic, and ribocil.

The past few years have led to substantial knowledge of the mechanisms behind functioning of noncoding RNAs like riboswitches and small RNAs (sRNA). Riboswitches play an important role in regulation of gene expression at the transcriptional or translation level.[34] They arise from the 5′ untranslated regions (UTRs) of the mRNA of the gene that they regulate. Riboswitches contain an aptamer domain (30−200 nucleotides) along with an expression platform. The expression platform has terminator sequences or anti-terminator sequences that are mutually exclusive and with different conformations. Upon binding of the ligand, it attains one of the conformations, thereby affecting transcription (Figure 7). The conformation change in the expression platform might also affect the ribosome binding at the ribosome binding site, thereby impeding translation. Riboswitches are present in most bacteria like *M. tuberculosis*, *Vibrio cholerae*, and *S. pneumoniae* but are absent in mammals, making them potential drug targets.[34] They regulate important cellular pathways, especially metabolic pathways, and multiple ligands controlling riboswitches have been classified. These ligands include certain amino acids, purines, thiamines, flavin mononucleotide (FMN), as well as c-di-GMP.[26,34] A riboflavin analogue called roseoflavin was discovered in 1974, but its underlying mechanism of FMN riboswitch inhibition was discovered much later in 2009.[35] This riboswitch controls the riboflavin (vitamin B2) biosynthesis pathway, which is a precursor to synthesizing cofactors like FMN. The pathway is essential in the absence of riboflavin in the environment, and is essential in *M. tuberculosis* as it lacks riboflavin transport genes. This inspired the subsequent discovery of another selective synthetic inhibitor of FMN riboswitch named "ribocil" (Figure 8).[36] Ribocil was found to interact with the FMN riboswitch at the same domain as FMN to shut down the riboflavin synthesis, despite their chemical structures being quite dissimilar to one another. Ribocil showed promising results in infected mice and decreased the bacterial load by 1000-fold. High-throughput screening strategies and structure-guided ligand docking have been used to identify small molecule inhibitors of riboswitches.[37] These screening strategies also require development of assays for riboswitch binding. Most often, this is achieved by using displacement assays involving displacement of a fluorescent ligand by a non-fluorescent one from the compound library. Chemical perturbation of the natural ligands or the potential small molecule candidates identified by such high-throughput screening is then done to increase their specificity and affinity. Further studies are needed to design and synthesize such small molecule inhibitors of other riboswitches as well so as to target other pathways essential for stress survival and pathogenesis.

sRNAs are regulatory RNAs that range from 40 to 500 nucleotides in length.[37] They can arise from 5′ or 3′ UTRs of mRNAs and also from transcription of dedicated genes.[38] Although they are not essential, they regulate pathways associated with biofilm formation, virulence, antibiotic resistance, and other stress-associated pathways.[39] sRNAs can be positive or negative regulators of gene expression and generally mediate their action by binding to a target mRNA in the presence of a RNA chaperone protein like Hfq. Antibiotics that inhibit transcription inhibit sRNA synthesis as well. However, sRNAs have a limited structural complexity making it difficult to design inhibitors for them. An alternative strategy to target sRNAs is to target the Hfq protein. sRNAs could also be modulated by antisense technology. Recently, screening a library of cyclic peptides led to the discovery of several in vitro inhibitors of Hfq activity.[40] This was achieved by employing a reporter assay with a Hfq−sRNA controlled expression of a fluorescent protein. With more sRNAs and RNA chaperones being characterized, this could lead to the discovery of potential drugs.

6. CONCLUSIONS

The current pace of antibiotic discovery is not sufficient to combat antibiotic resistance, necessitating the development of alternate strategies. In this regard, antibacterial compounds that are not standalone drugs but are synergistic with the conventional antibiotics would be a step forward. Most antibiotics either target the three processes of the central dogma or the cell wall metabolism, and there is a need to look beyond these processes for identifying new drug targets. Attacking the stress-associated pathways is therefore a promising strategy for eliminating infections. Chemical design and synthesis of structural analogues of signaling nucleotides like (p)ppGpp is one such way. sRNAs and riboswitches governing stress-related pathways can be targeted by rational design of small molecule inhibitors. Peptide inhibitors of biofilms have also shown impressive results in killing antibiotic-resistant strains. Additionally, several drugs targeting metabolism and respiration are in clinical trials.[41] Improving respiration in *M. tuberculosis* persister cells by adding thiols led to the cells becoming metabolically active and hence drug-susceptible.[42] *M. tuberculosis* experiences acid stress inside phagosomes and a diamide derivative AC2P36 has been synthesized that kills *M. tuberculosis* at low pH by affecting thiol stress.[43] Another example of a non-essential protein proven to be a good drug candidate is the enzyme tryptophan synthase against which a small molecule allosteric inhibitor (a synthetic azetidine derivative) has shown remarkable results in *M. tuberculosis*.[44] Although these studies were performed in *M. tuberculosis*,

DOI: 10.1021/acsomega.7b01368
ACS Omega 2017, 2, 7400−7409

similar strategies could be applicable in other pathogenic bacteria as well. An alternate approach is to improve efficacy of current antibiotics by chemical modification or other means like addition of adjuvants. Drug accumulation inside bacteria varies according to cell wall properties as well as physicochemical properties of the drug, and therefore, a systematic analysis of a large number of compounds is useful in drug design and modification.[45] Spiroisoxazoline molecules called "small molecules aborting resistance" (SMARt) have been recently developed that activate alternate pathways of the prodrug ethionamide, thereby reverting acquired resistance in *M. tuberculosis*.[46] Genome and metabolome data of several clinical isolates of pathogenic strains have shed more light on mechanisms of antibiotic resistance and revealed new cellular pathways as potential drug targets. Rationalized chemical design of inhibitors of such pathways should lead to development of more drugs in the near future.

AUTHOR INFORMATION

Corresponding Author

*E-mail: dipankar@iisc.ac.in. Phone: +91-80-22932836.

ORCID

Dipankar Chatterji: 0000-0002-9985-0632

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

A.P. is thankful to Indian Institute of Science, Bangalore, for fellowship. D.C. acknowledges financial support from Department of Science and Technology, Government of India.

REFERENCES

(1) World Health Organization. *Antibacterial Agents in Clinical Development: An Analysis of the Antibacterial Clinical Development Pipeline, Including Tuberculosis*; WHO: Geneva, 2017, WHO/EMP/IAU/2017.12.

(2) Fleming, A. On the antibacterial action of cultures of a Penicillium, with special reference to their use in the isolation of *B. influenzae*. *Br. J. Exp. Pathol.* **1929**, *10*, 226−236.

(3) Brown, E. D.; Wright, G. D. Antibacterial drug discovery in the resistance era. *Nature* **2016**, *529*, 336−343.

(4) Blair, J. M.; Webber, M. A.; Baylay, A. J.; Ogbolu, D. O.; Piddock, L. J. Molecular mechanisms of antibiotic resistance. *Nat. Rev. Microbiol.* **2015**, *13*, 42−51.

(5) Rice, L. B. Federal funding for the study of antimicrobial resistance in nosocomial pathogens: no ESKAPE. *J. Infect. Dis.* **2008**, *197*, 1079−1081.

(6) Kerantzas, C. A.; Jacobs, W. R., Jr. Origins of Combination Therapy for Tuberculosis: Lessons for Future Antimicrobial Development and Application. *mBio* **2017**, *8*, No. e01586.

(7) Smith, T.; Wolff, K. A.; Nguyen, L. Molecular biology of drug resistance in *Mycobacterium tuberculosis*. *Curr. Top. Microbiol. Immunol.* **2013**, *374*, 53−80.

(8) Katayama, Y.; Ito, T.; Hiramatsu, K. A new class of genetic element, staphylococcus cassette chromosome mec, encodes methicillin resistance in *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **2000**, *44*, 1549−1555.

(9) Hegde, S. S.; Vetting, M. W.; Roderick, S. L.; Mitchenall, L. A.; Maxwell, A.; Takiff, H. E.; Blanchard, J. S. A fluoroquinolone resistance protein from *Mycobacterium tuberculosis* that mimics DNA. *Science* **2005**, *308*, 1480−1483.

(10) Burian, J.; Ramon-Garcia, S.; Sweet, G.; Gomez-Velasco, A.; Av-Gay, Y.; Thompson, C. J. The mycobacterial transcriptional regulator whiB7 gene links redox homeostasis and intrinsic antibiotic resistance. *J. Biol. Chem.* **2012**, *287*, 299−310.

(11) Cohen, N. R.; Lobritz, M. A.; Collins, J. J. Microbial persistence and the road to drug resistance. *Cell Host Microbe* **2013**, *13*, 632−642.

(12) Harms, A.; Maisonneuve, E.; Gerdes, K. Mechanisms of bacterial persistence during stress and antibiotic exposure. *Science* **2016**, *354*, No. aaf4268.

(13) Nguyen, D.; Joshi-Datar, A.; Lepine, F.; Bauerle, E.; Olakanmi, O.; Beer, K.; McKay, G.; Siehnel, R.; Schafhauser, J.; Wang, Y.; Britigan, B. E.; Singh, P. K. Active starvation responses mediate antibiotic tolerance in biofilms and nutrient-limited bacteria. *Science* **2011**, *334*, 982−986.

(14) Maisonneuve, E.; Castro-Camargo, M.; Gerdes, K. (p)ppGpp controls bacterial persistence by stochastic induction of toxin-antitoxin activity. *Cell* **2013**, *154*, 1140−1150.

(15) Amato, S. M.; Orman, M. A.; Brynildsen, M. P. Metabolic control of persister formation in *Escherichia coli*. *Mol. Cell* **2013**, *50*, 475−487.

(16) Costerton, J. W.; Stewart, P. S.; Greenberg, E. P. Bacterial biofilms: a common cause of persistent infections. *Science* **1999**, *284*, 1318−1322.

(17) Weiss, L. A.; Stallings, C. L. Essential roles for *Mycobacterium tuberculosis* Rel beyond the production of (p)ppGpp. *J. Bacteriol.* **2013**, *195*, 5629−5638.

(18) Hauryliuk, V.; Atkinson, G. C.; Murakami, K. S.; Tenson, T.; Gerdes, K. Recent functional insights into the role of (p)ppGpp in bacterial physiology. *Nat. Rev. Microbiol.* **2015**, *13*, 298−309.

(19) Wexselblatt, E.; Oppenheimer-Shaanan, Y.; Kaspy, I.; London, N.; Schueler-Furman, O.; Yavin, E.; Glaser, G.; Katzhendler, J.; Ben-Yehuda, S. Relacin, a novel antibacterial agent targeting the Stringent Response. *PLoS Pathog.* **2012**, *8*, No. e1002925.

(20) Wexselblatt, E.; Kaspy, I.; Glaser, G.; Katzhendler, J.; Yavin, E. Design, synthesis and structure-activity relationship of novel Relacin analogs as inhibitors of Rel proteins. *Eur. J. Med. Chem.* **2013**, *70*, 497−504.

(21) Syal, K.; Flentie, K.; Bhardwaj, N.; Maiti, K.; Jayaraman, N.; Stallings, C. L.; Chatterji, D. Synthetic (p)ppGpp Analogue Is an Inhibitor of Stringent Response in Mycobacteria. *Antimicrob. Agents Chemother.* **2017**, *61*, No. e00443.

(22) Vilchèze, C.; Hartman, T.; Weinrick, B.; Jacobs, W. R., Jr. *Mycobacterium tuberculosis* is extraordinarily sensitive to killing by a vitamin C-induced Fenton reaction. *Nat. Commun.* **2013**, *4*, No. 1881.

(23) Syal, K.; Bhardwaj, N.; Chatterji, D. Vitamin C targets (p)ppGpp synthesis leading to stalling of long-term survival and biofilm formation in *Mycobacterium smegmatis*. *FEMS Microbiol. Lett.* **2017**, *364*, No. fnw282.

(24) Gaca, A. O.; Kudrin, P.; Colomer-Winter, C.; Beljantseva, J.; Liu, K.; Anderson, B.; Wang, J. D.; Rejman, D.; Potrykus, K.; Cashel, M.; Hauryliuk, V.; Lemos, J. A. From (p)ppGpp to (pp)pGpp: Characterization of Regulatory Effects of pGpp Synthesized by the Small Alarmone Synthetase of *Enterococcus faecalis*. *J. Bacteriol.* **2015**, *197*, 2908−2919.

(25) Beljantseva, J.; Kudrin, P.; Andresen, L.; Shingler, V.; Atkinson, G. C.; Tenson, T.; Hauryliuk, V. Negative allosteric regulation of *Enterococcus faecalis* small alarmone synthetase RelQ by single-stranded RNA. *Proc. Natl. Acad. Sci. U.S.A.* **2017**, *114*, 3726−3731.

(26) Hengge, R.; Grundling, A.; Jenal, U.; Ryan, R.; Yildiz, F. Bacterial Signal Transduction by Cyclic Di-GMP and Other Nucleotide Second Messengers. *J. Bacteriol.* **2016**, *198*, 15−26.

(27) O'Toole, G.; Kaplan, H. B.; Kolter, R. Biofilm formation as microbial development. *Annu. Rev. Microbiol.* **2000**, *54*, 49−79.

(28) Pletzer, D.; Hancock, R. E. Antibiofilm Peptides: Potential as Broad-Spectrum Agents. *J. Bacteriol.* **2016**, *198*, 2572−2578.

(29) Overhage, J.; Campisano, A.; Bains, M.; Torfs, E. C.; Rehm, B. H.; Hancock, R. E. Human host defense peptide LL-37 prevents bacterial biofilm formation. *Infect. Immun.* **2008**, *76*, 4176−4782.

(30) Haney, E. F.; Mansour, S. C.; Hilchie, A. L.; de la Fuente-Nunez, C.; Hancock, R. E. High throughput screening methods for assessing antibiofilm and immunomodulatory activities of synthetic peptides. *Peptides* **2015**, *71*, 276−285.

DOI: 10.1021/acsomega.7b01368
ACS Omega 2017, 2, 7400−7409

(31) de la Fuente-Núñez, C.; Reffuveille, F.; Mansour, S. C.; Reckseidler-Zenteno, S. L.; Hernandez, D.; Brackman, G.; Coenye, T.; Hancock, R. E. D-enantiomeric peptides that eradicate wild-type and multidrug-resistant biofilms and protect against lethal *Pseudomonas aeruginosa* infections. *Chem. Biol.* **2015**, *22*, 196−205.
(32) Andresen, L.; Tenson, T.; Hauryliuk, V. Cationic bactericidal peptide 1018 does not specifically target the stringent response alarmone (p)ppGpp. *Sci. Rep.* **2016**, *6*, No. 36549.
(33) Silva, T.; Moreira, A. C.; Nazmi, K.; Moniz, T.; Vale, N.; Rangel, M.; Gomes, P.; Bolscher, J. G. M.; Rodrigues, P. N.; Bastos, M.; Gomes, M. S. Lactoferricin Peptides Increase Macrophages' Capacity To Kill Mycobacterium avium. *mSphere* **2017**, *2*, No. e00301.
(34) Serganov, A.; Nudler, E. A decade of riboswitches. *Cell* **2013**, *152*, 17−24.
(35) Lee, E. R.; Blount, K. F.; Breaker, R. R. Roseoflavin is a natural antibacterial compound that binds to FMN riboswitches and regulates gene expression. *RNA Biol.* **2009**, *6*, 187−194.
(36) Howe, J. A.; Wang, H.; Fischmann, T. O.; Balibar, C. J.; Xiao, L.; Galgoci, A. M.; Malinverni, J. C.; Mayhood, T.; Villafania, A.; Nahvi, A.; Murgolo, N.; Barbieri, C. M.; Mann, P. A.; Carr, D.; Xia, E.; Zuck, P.; Riley, D.; Painter, R. E.; Walker, S. S.; Sherborne, B.; de Jesus, R.; Pan, W.; Plotkin, M. A.; Wu, J.; Rindgen, D.; Cummings, J.; Garlisi, C. G.; Zhang, R.; Sheth, P. R.; Gill, C. J.; Tang, H.; Roemer, T. Selective small-molecule inhibition of an RNA structural element. *Nature* **2015**, *526*, 672−677.
(37) Colameco, S.; Elliot, M. A. Non-coding RNAs as antibiotic targets. *Biochem. Pharmacol.* **2017**, *133*, 29−42.
(38) Miyakoshi, M.; Chao, Y.; Vogel, J. Regulatory small RNAs from the 3′ regions of bacterial mRNAs. *Curr. Opin. Microbiol.* **2015**, *24*, 132−139.
(39) Michaux, C.; Hartke, A.; Martini, C.; Reiss, S.; Albrecht, D.; Budin-Verneuil, A.; Sanguinetti, M.; Engelmann, S.; Hain, T.; Verneuil, N.; Giard, J. C. Involvement of *Enterococcus faecalis* small RNAs in stress response and virulence. *Infect. Immun.* **2014**, *82*, 3599−3611.
(40) El-Mowafi, S. A.; Alumasa, J. N.; Ades, S. E.; Keiler, K. C. Cell-based assay to identify inhibitors of the Hfq-sRNA regulatory pathway. *Antimicrob. Agents Chemother.* **2014**, *58*, 5500−5509.
(41) Bald, D.; Villellas, C.; Lu, P.; Koul, A. Targeting Energy Metabolism in *Mycobacterium tuberculosis*, a New Paradigm in Antimycobacterial Drug Discovery. *mBio* **2017**, *8*, No. e00272.
(42) Vilchèze, C.; Hartman, T.; Weinrick, B.; Jain, P.; Weisbrod, T. R.; Leung, L. W.; Freundlich, J. S.; Jacobs, W. R., Jr. Enhanced respiration prevents drug tolerance and drug resistance in *Mycobacterium tuberculosis*. *Proc. Natl. Acad. Sci. U.S.A.* **2017**, *114*, 4495−4500.
(43) Coulson, G. B.; Johnson, B. K.; Zheng, H.; Colvin, C. J.; Fillinger, R. J.; Haiderer, E. R.; Hammer, N. D.; Abramovitch, R. B. Targeting Mycobacterium tuberculosis Sensitivity to Thiol Stress at Acidic pH Kills the Bacterium and Potentiates Antibiotics. *Cell Chem. Biol.* **2017**, *24*, No. 993.e4.
(44) Wellington, S.; Nag, P. P.; Michalska, K.; Johnston, S. E.; Jedrzejczak, R. P.; Kaushik, V. K.; Clatworthy, A. E.; Siddiqi, N.; McCarren, P.; Bajrami, B.; Maltseva, N. I.; Combs, S.; Fisher, S. L.; Joachimiak, A.; Schreiber, S. L.; Hung, D. T. A small-molecule allosteric inhibitor of *Mycobacterium tuberculosis* tryptophan synthase. *Nat. Chem. Biol.* **2017**, *13*, 943−950.
(45) Richter, M. F.; Drown, B. S.; Riley, A. P.; Garcia, A.; Shirai, T.; Svec, R. L.; Hergenrother, P. J. Predictive compound accumulation rules yield a broad-spectrum antibiotic. *Nature* **2017**, *545*, 299−304.
(46) Blondiaux, N.; Moune, M.; Desroses, M.; Frita, R.; Flipo, M.; Mathys, V.; Soetaert, K.; Kiass, M.; Delorme, V.; Djaout, K.; Trebosc, V.; Kemmer, C.; Wintjens, R.; Wohlkonig, A.; Antoine, R.; Huot, L.; Hot, D.; Coscolla, M.; Feldmann, J.; Gagneux, S.; Locht, C.; Brodin, P.; Gitzinger, M.; Deprez, B.; Willand, N.; Baulard, A. R. Reversion of antibiotic resistance in *Mycobacterium tuberculosis* by spiroisoxazoline SMARt-420. *Science* **2017**, *355*, 1206−1211.

DOI: 10.1021/acsomega.7b01368
ACS Omega 2017, 2, 7400−7409