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PERSPECTIVE

Riboswitches as therapeutic targets: promise of a new era of antibiotics

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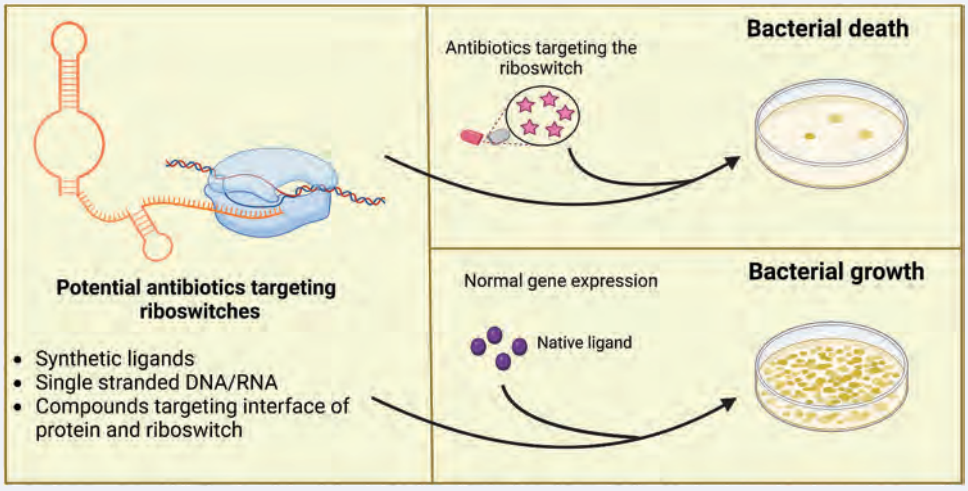
ABSTRACT

Introduction: The growth of antibiotic resistance among bacterial pathogens is an impending global threat that can only be averted through the development of novel antibacterial drugs. A promising answer could be the targeting of riboswitches, structured RNA elements found almost exclusively in bacteria.

Areas covered: This review examines the potential of riboswitches as novel antibacterial drug targets. The limited mechanisms of action of currently available antibiotics are summarized, followed by a delineation of the functional mechanisms of riboswitches. We then discuss the potential for developing novel approaches that target paradigmatic riboswitches in the context of their bacterial gene expression machinery.

Expert opinion: We highlight potential advantages of targeting riboswitches in their functional form, embedded within gene expression complexes critical for bacterial survival. We emphasize the benefits of this approach, including potentially higher species specificity and lower side effects.



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Antibiotics; bacteria; drug target; riboswitches; translation; transcription

1. Introduction: the threat of antibiotic resistance

Over the past century, the vast improvement of healthcare and related sectors is partly attributed to the development of antibiotics, which contributed to an increase in the global life expectancy from 32 to 72.6 years from 1900 until the present day [1]. Unfortunately, the rise of bacterial drug resistance is now posing a serious public health threat to this achievement of modern science. Antibiotic resistance is a naturally occurring, evolutionary process in which over time bacteria adapt to and withstand the effects of antibiotics. This evolution has been accelerated by the misuse of antibiotics through over-prescription, patient noncompliance with prescription guidelines, and the use of clinical antibiotics in animal agriculture [2]. The resulting rapid growth of antibiotic resistance has led to the spread of 'superbugs' such as Methicillin-resistant *Staphylococcus aureus* (MRSA) and *Clostridium difficile* (*C. difficile*). In 2019 alone, an estimated 1.27 million deaths were attributed to antimicrobial resistance with over 100,000 of those resulting directly from MRSA [3]. Accordingly, the World Health Organization (WHO) has classified antibiotic resistance as one of the major threats to global health and food security [4], and the US Centers for Disease Control and Prevention (CDC) list many pathogens as 'urgent' or 'serious' threats [5]. Reminded of the devastating effects of a global pandemic, so recently caused by the SARS-CoV-2 virus, now renders taking action against the expected bacterial 'shadow pandemic' even more urgent [6].

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Article highlights

- Antibiotic resistance is a growing worldwide public health threat that demands the development of novel antibacterial drugs.
- Riboswitches are predominantly found within bacteria where they control gene expression through transcription or translation, offering an unprecedented target for antibacterials.
- Riboswitches are broadly found within most high priority pathogens listed by the CDC and WHO.
- The lysine, FMN, TPP, and guanine riboswitches are paradigms that show promise for developing antibacterials based on either small molecules or RNA therapeutics.
- The interdependence and physical proximity of riboswitches and conserved gene expression complexes involving the bacterial RNA polymerase and ribosome offers an opportunity for developing antibiotics of either broad or narrow specificity.

The current crisis of antimicrobial resistance has been amplified by the relative paucity of new drug development [2]. One of the major challenges facing the development of new antibacterials is the lack of financial incentives. The short treatment course during acute infection combined with the long and expensive development process compares unfavorably for the pharmaceutical industry with the profitability of drugs against chronic diseases. Consequently, the allocation of resources toward new antibiotics is vastly underwhelming. One striking example is the United Kingdom, which despite being one of the largest investors of antimicrobial resistance research among JPIAMR (Joint Programming Initiative on Antimicrobial Resistance) countries, allocated a mere 1% of its total research funding toward this crucial goal between 2007 and 2013 [7]. The US government has become increasingly creative to provide stronger incentives, including the 2012 Generating Antibiotic Incentives Now Act; the 2016 implementation of the nonprofit public–private partnership CARB-X; the launch in 2016 of the 21st Century Cures Act; and the push for a drug development subscription program through the 2020 Pioneering Antimicrobial Subscriptions to End Upsurging Resistance or Pasteur Act, currently debated in a gridlocked congress [8]. So far, none of these efforts has made a significant difference in the goal of staying ahead of bacterial evolution so that the need for critical government support for antibiotics research remains. If antimicrobial resistance is not sufficiently confronted, it is estimated that 10 million annual deaths worldwide will occur by 2050, with a total healthcare cost of up to 100 trillion dollars, more than the yearly GDP of most countries [9].

Current antibacterial agents function through five major mechanisms, including through the disruption of membrane structure, inhibition of specific metabolic pathways, or of cell wall, DNA or protein biosynthesis, with a majority of antibiotics targeting the ribosome while translating messenger RNAs (mRNAs) into protein [10]. Bacteria have demonstrated the ability to develop resistance against various modes of antibacterial action. This includes instances such as β-lactamase enzymes inactivating antibiotics that would usually prevent cell wall biosynthesis to changes in bacterial membrane proteins to reduce the amount of antibiotics passing into the bacterium or to accelerate drug export [10]. Consequently, the development of new antibacterial modes of action has to be a worldwide priority.

One underexplored mechanism for expanding the clinical arsenal of antibiotics is the targeting of riboswitches.

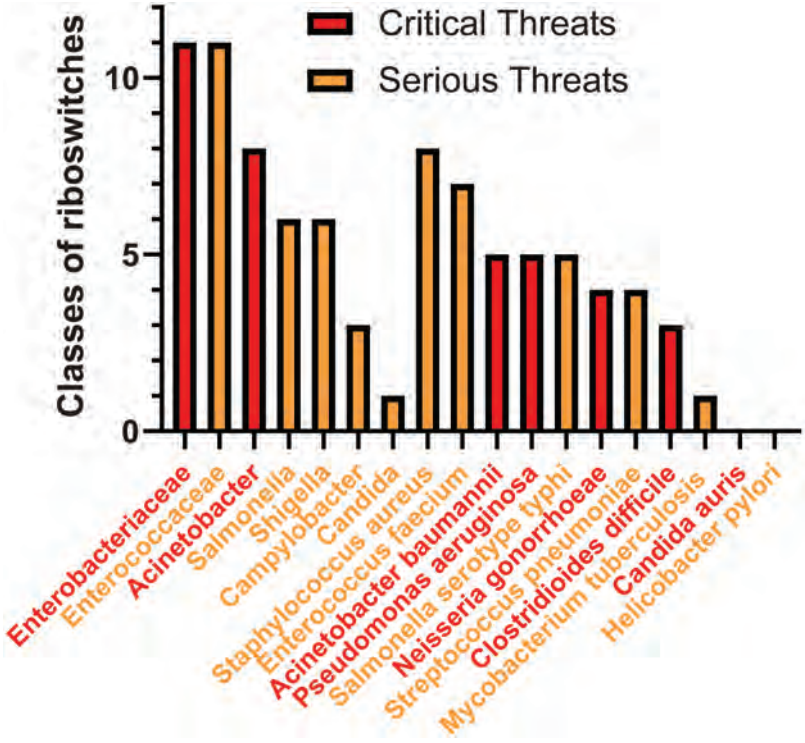

Figure 1. The frequency of riboswitch classes found within the high priority pathogens as identified by the WHO and/or CDC based on their drug resistance. Critical threats are shown in red and serious threats are shown in orange. Pathogens are listed from left to right in the order of family, genus, and species.

Table 1. High priority pathogens as identified by the WHO and/or CDC based on their drug-resistance, and the riboswitches within those families or species.

Threat level	Genus/Family	Riboswitches
CDC Urgent Threat	Acinetobacter (–)	AdoCbl, Fluoride, FMN, Glycine, Guanidine-I, Guanidine-II, SAH, TPP
CDC Serious Threat, WHO High Threat	Campylobacter (–)	Guanidine-I, FMN, TPP
CDC Serious Threat	Candida (+)	TPP
CDC Urgent Threat, WHO Critical Threat	Enterobacteriaceae (–)	Fluoride, FMN, Glycine, Guanidine-I/II, Lysine, Magnesium, Manganese, Moco, TPP, ZTP
CDC Serious Threat	Enterococcaceae (+)	AdoCbl, Fluoride, FMN, GlcN6P, Glycine, Guanidine-I, Lysine, PreQ1-I, PreQ1-II, THF, TPP
CDC Serious Threat, WHO High Threat	Salmonella (–)	FMN, Guanidine-II, Magnesium, Manganese, Moco, TPP
CDC Serious Threat	Shigella (–)	FMN, Guanidine-II, Lysine, Magnesium, Moco, TPP
WHO Critical Threat	Acinetobacter baumannii (–)	Fluoride, FMN, Glycine, Manganese, TPP
CDC Urgent Threat	Candida auris (–)	?
CDC Urgent Threat	Clostridioides difficile (+)	C-di-GMP-I, C-di-GMP-II, FMN
WHO High Threat	Enterococcus faecium (+)	Fluoride, FMN, GlcN6P, Lysine, PreQ1, THF, TPP
WHO High Threat	Helicobacter pylori (–)	?
CDC Serious Threat	Mycobacterium tuberculosis (+)	Glycine
CDC Urgent Threat, WHO High Threat	Neisseria gonorrhoeae (–)	Glycine, SAM-I/IV, PreQ1, TPP
WHO Critical Threat, CDC Serious Threat	Pseudomonas aeruginosa (–)	Fluoride, FMN, Guanidine-II, Manganese, TPP
CDC Serious Threat	Salmonella serotype typhi (–)	AdoCbl, FMN, Magnesium, Moco, TPP
CDC Serious Threat, WHO High Threat	Staphylococcus aureus (+)	FMN, GlcN6P, Glycine, Guanidine-I, Lysine, Manganese, SAM, TPP
CDC Serious Threat	Streptococcus pneumoniae (+)	FMN, Glycine, PreQ1-II, TPP

Abbreviations: (–), gram negative; (+), gram positive; ?, no riboswitches known yet; AdoCbl, Adenosylcobalamin or coenzyme B12; C-di-GMP, Cyclic-di-GMP; Moco, molybdenum cofactor; ZTP, 5-aminoimidazole-4-carboxamide ribonucleosides-5′-triphosphate; GlcN6P, Glucosamine-6-phosphate activated ribozyme; THF, tetrahydrofolate, SAM, S-adenosylmethionine.

Riboswitches are widespread RNA structural motifs found in the 5′ untranslated regions (5′ UTRs) of mRNAs that in bacteria, including many that are pathogenic (Figure 1 and Table 1), function to regulate transcription, translation, or RNA decay [11,12]. In some bacteria, riboswitches control more than 4% of genes, including those coding for many essential cell products [13]. Two major mechanisms riboswitches exploit to regulate gene expression involve conformational changes that either cause bacterial RNA polymerase (RNAP) to end transcription or prevent translation initiation by sequestering the ribosome binding site [14,15]. Since the transcription and translation machineries are highly conserved among bacteria, but riboswitches are absent in mammals, the application of a drug that targets a riboswitch or a riboswitch-protein interface promise to knock out necessary gene expression across bacterial species while leaving human cells unaffected. Conversely, the diversity of RNA sequences of related riboswitches among bacteria may also offer an angle to target specific pathogens, without harming the beneficial microbiome [16,17].

This review first details the mechanisms of antibiotics currently on the market, then outlines how antibacterials designed to target riboswitches could improve the drug effectiveness while retaining high specificity against pathogenic bacterial strains.

2. Antibiotic classes currently on the market

Currently available antibiotics interfere with vital biological pathways, thereby leading to bacterial cell death to treat acute infections. The historically first example of an antibiotic was Penicillin, a type of β-lactam antibiotic discovered in 1928 by Scottish scientist Alexander Fleming in the mold *Penicillium notatum* to fend off bacterial competitors. Fleming thus laid the roots for the 'miracle drug' that in 1942 pulled Anne Miller, a 33-year-old patient in a Connecticut hospital, back from certain death after a streptococcal infection following a miscarriage [18]. By 1945, US manufacturers were producing 646 billion 'units' – a standard adult dose today is between 200,000 and 500,000 units – of the new wonder drug every month, saving thousands of soldiers from an excruciating death by infection during World War II. The same year, Fleming shared into the Nobel Prize in Physiology or Medicine [18]. β-lactam antibiotics interfere with enzymes that are essential to synthesizing the bacterial cell wall [19,20].

Many antibiotics, often similarly initially isolated from fungi, have aimed at targeting bacterial translation and transcription. Among these processes, the bacterial ribosome is a major target for antibiotic development, through either its small 30S or large 50S subunit [10,21]. For the 30S subunit, antibiotics such as streptomycin or doxycycline typically inhibit or interfere with tRNA binding or the ability of tRNAs to move through the assembled ribosome [21]. Complementarily, antibiotics targeting the 50S subunit such as clindamycin or erythromycin redistribute or inhibit the loading of charged tRNAs or the movement of the nascent polypeptide chain through the ribosomal tunnel [21]. By comparison, fewer antibiotics target transcription, but one of significance is rifamycin, which inhibits the bacterial RNA polymerase and has been used to treat tuberculosis [22]. Finally, a recent, still less commonly used treatment is bacteriophage therapy wherein a naturally occurring virus (phage) is used to infect a bacterium. Following injection and replication of its phage genome in the cell, new phage particles form, leading to the lysis of the bacterium and autonomous phage spreading across a large bacterial population [23].

Today, the challenge of antibiotic resistance is decreasing the effectiveness of many commercial antibiotics. For example, rifamycins were given in combination with other antibiotics to increase the potency and shorten the length of a treatment, until single (and sometimes double) point mutations in the rpoB gene encoding the RNAP's beta subunit of clinical *Mycobacterium tuberculosis* strains altered the drug's binding site and led to resistance [10,22,24,25]. As antibiotic resistance continues to grow, there is a critical need for innovative strategies and novel solutions to overcome this pressing public health threat.

Next, we will discuss the potential of riboswitches to serve as antibacterial targets.

3. Riboswitches as a unique Achilles' heel of bacteria

3.1. Classes and functional mechanisms of riboswitches

Riboswitches are structured non-coding RNA elements commonly embedded in the 5′ UTRs of mRNA that regulate downstream genes in response to the binding of small molecules or ions. They are important for efficient resource allocation during times of stress and changing environmental conditions [26]. Riboswitches consist of two domains, an aptamer domain that binds a ligand(s) and an expression platform involved in the regulation of gene expression. The binding of a ligand to the aptamer domain triggers a conformational change within the expression platform (which are often overlapping) that either terminates or promotes the expression of downstream genes [11,27,28] (Figure 2). Currently, 55 classes of riboswitches are known, distinguished by the specific ligand they bind, with many more 'boutique' classes expected to be found in individual bacterial strains adapted to unique environmental niches [29]. Riboswitches are further stratified based on their mechanism of regulation; most control gene expression at the level of transcription or translation (Figure 2), which can eventually lead to RNA degradation [11,12,27]. Intriguingly, transcriptional riboswitches are found primarily in Gram-positive bacteria. In contrast, translational riboswitches are confined mainly to Gram-negative bacteria that also contain the conserved Rho protein, which serves as a transcription terminator. Translational riboswitches have been found to trigger Rho-dependent transcription termination thus regulating both transcription and translation in Gram-negative bacteria [30].

Riboswitches are further characterized by their effect on downstream genes upon ligand binding; OFF-switches terminate transcription or translation of a gene encoded by the mRNA upon binding of their ligand, whereas ON-switches promote the expression of downstream genes [31]. Most OFF-switches regulate genes related to the production of essential metabolites so that the termination of gene expression can be exploited as antibacterial drug target, while ON-switches commonly regulate genes involved in the production of export proteins for their cytotoxic ligands.

In the following, we focus on the potential of riboswitches as novel antibacterial targets before we turn to specific riboswitches whose functional disruption by drugs has been shown to result in bacterial cell death.

3.2. Riboswitches offer a new avenue for antibiotic drug development

Most antibiotics have been developed against protein targets (exception being those targeting the ribonucleoprotein complex of the ribosome); however, as we look toward

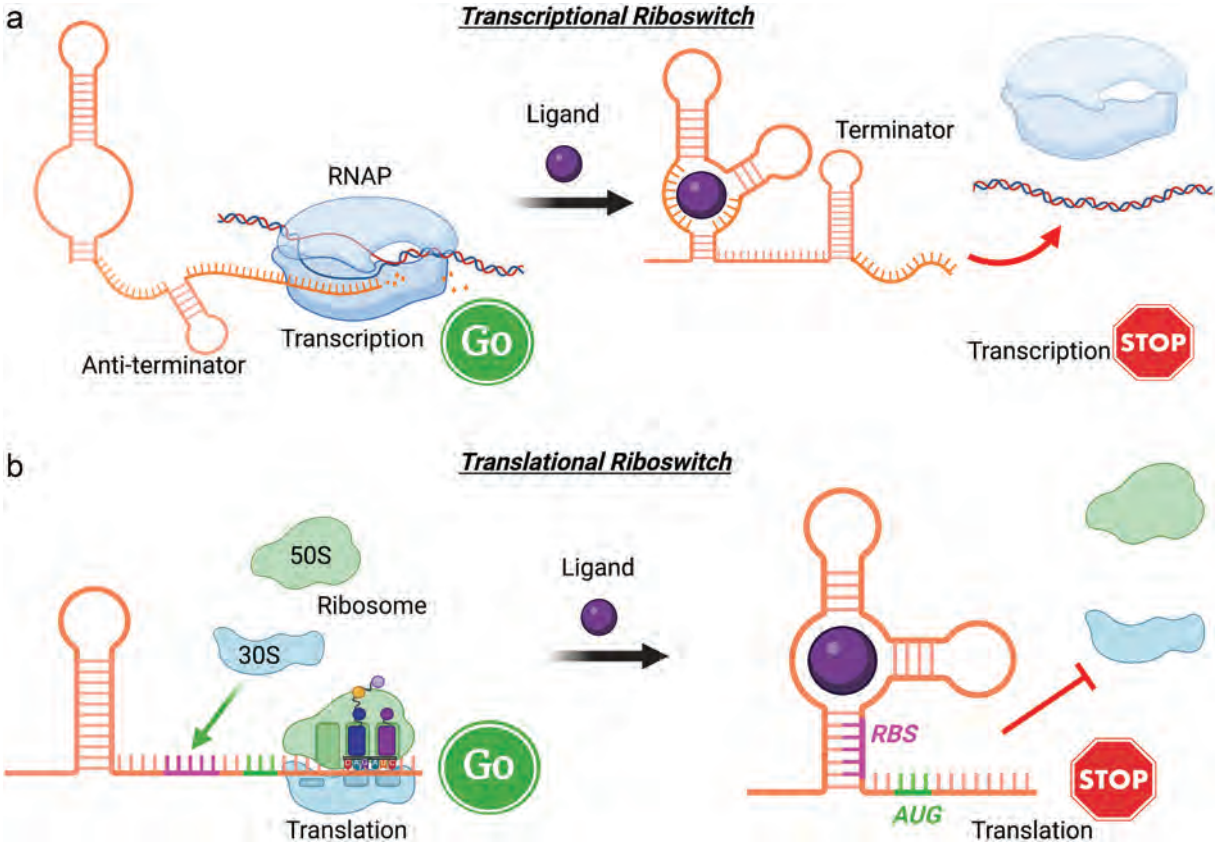

Figure 2. Riboswitches can regulate bacterial gene expression at either the level of transcription or translation. Upon binding of a ligand to the aptamer domain of a riboswitch there is a conformational change in the expression platform. In transcriptional OFF-switches, the RNA polymerase (RNAP) is dislodged, leading to transcription termination. In translational OFF-switches, the ribosome binding site (RBS), shown in pink, is sequestered, preventing ribosome binding and translation. Figure created with BioRender.

finding novel, innovative strategies for drug discovery, RNA targets may hold the key to a new era of antibiotics [32]. Specifically, most high-priority pathogens listed by the CDC and WHO contain riboswitches (Figure 1 and Table 1) [5,33], with the thiamine pyrophosphate (TPP) riboswitch alone found in nearly all of them [18]. For example, MRSA is a 'serious' threat recognized by the CDC and known to contain multiple riboswitches, including those binding TPP, lysine, and flavin mononucleotide (FMN) [18].

Unlike the two fully complementary strands of DNA, RNA is normally created as a single strand that can form intricate secondary and tertiary structures, allowing for an extensive range of functions that offer target potential for designing antibiotics [34]. Since riboswitches are predominant in bacteria, but absent in mammals (as far as we know), manipulating gene expression through riboswitches is a particularly promising strategy for antibacterial drug development [35–38]. Riboswitches highly selectively bind a diverse range of ligands including amino acids, coenzymes, nucleotide derivatives, metal cations, and halide anions [31,39,40], demonstrating that they can bind small drug-like molecules. They are essential for maintaining the biological viability of microbes in changing environments as gene regulation in response to specific levels of ligand in the cell is crucial for maintaining viability [29].

The major advantage of drug-targeting riboswitches is that they are not found in higher eukaryotes, except for the TPP riboswitch involved in alternative splicing in plants and fungi [29], so that drugs targeting them are expected to exhibit relatively low mammalian cytotoxicity [41,42]. Riboswitches in principle can be targeted in multiple ways, including with synthetic small molecules that mimic the native ligand effect and thus trigger gene regulation independently of the cell's needs; by blocking their ribosome binding site with a single-stranded antisense RNA; or by disrupting interactions between the riboswitch and its protein partners (Figure 3). In addition to targeting the RNAP or ribosome as illustrated by the mechanisms of many antibiotics on the market (Figure 3 (b)), gene expression could also be disrupted at the interface of riboswitches and the transcription or translation machinery.

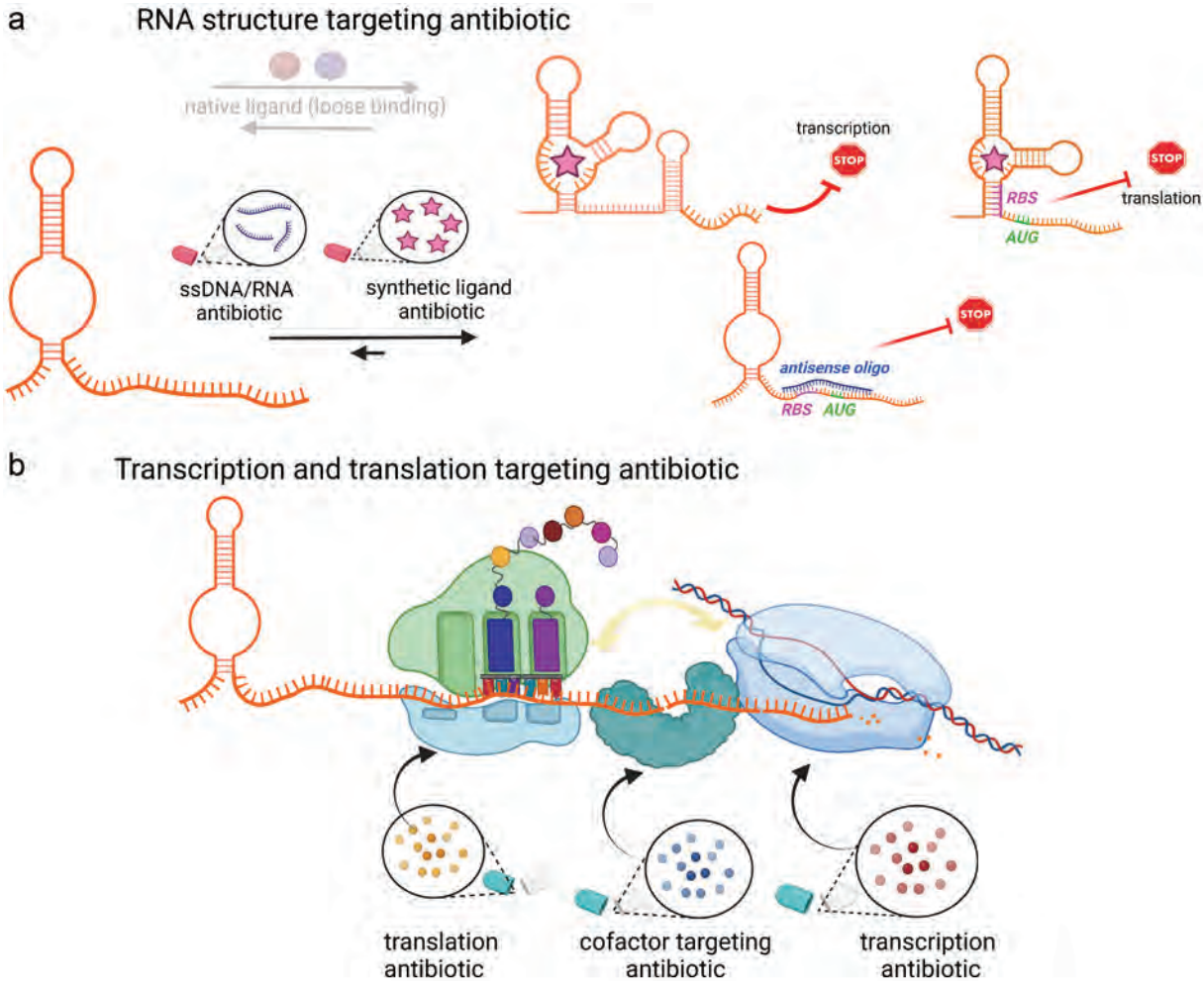

Figure 3. Mechanism of targeting riboswitches with antibiotics to interfere with the transcription and/or translation machinery. (a) Both transcriptional and translational riboswitches can be targeted with synthetic ligand mimics to prevent gene expression by dislodging the RNAP or sequestering the RBS. Additionally, antisense oligonucleotides can be designed to sequester the ribosome binding site of translational riboswitches to prevent translation initiation. (b) Antibiotics have traditionally been used to target protein complexes such as the ribosome, protein cofactors, or RNAP. The interfaces between these protein complexes (yellow double-arrow) and their riboswitches could be targeted with novel antibiotics. Figure created with BioRender.

Progress has been made particularly in the first modality of small molecules, such as vitamin and amino acids derivatives that inhibit bacterial growth by targeting riboswitches [37,41,43]. For example, roseoflavin, L-aminoethylcysteine and DL-4-oxalysine have been found to inhibit Gram-positive bacteria by repressing genes regulated by riboswitches that change conformation upon binding chemically related metabolite and cofactor ligands [43], whereas ribocil and its derivatives target the FMN riboswitch and so inhibit Gram-negative bacteria [44,45]. These are all cases of a ligand mimic that binds to a riboswitch, through which it is possible to suppress the expression of genes essential for bacterial growth (Figure 3a).

In the following, we highlight transcriptional riboswitches targeted in antibiotic development, where a synthetic ligand mimic has been found to successfully deregulate downstream gene transcription and thus inhibit bacterial growth.

3.3. The TPP riboswitch

The TPP riboswitch regulates the expression of thiamine metabolism genes upon binding thiamine pyrophosphate, a derivative of vitamin B1 [41,46,47]. Since thiamine is an essential cofactor for central metabolic pathways such as sugar and protein biosynthesis, this RNA can be a potential target for antibacterial drug development [41]. The TPP riboswitch is the most abundant riboswitch found in bacteria as well as the only one discovered (so far) in eukaryotes. Bacterial TPP riboswitches typically control gene expression at the level of transcription, where the binding of TPP triggers a conformational change in the riboswitch that suppresses transcription [41,47,48] (Figure 4(a)).

Several analogs of thiamine, including pyrithiamine (PT) and pyrithiamine pyrophosphate (PTPP), have been investigated for

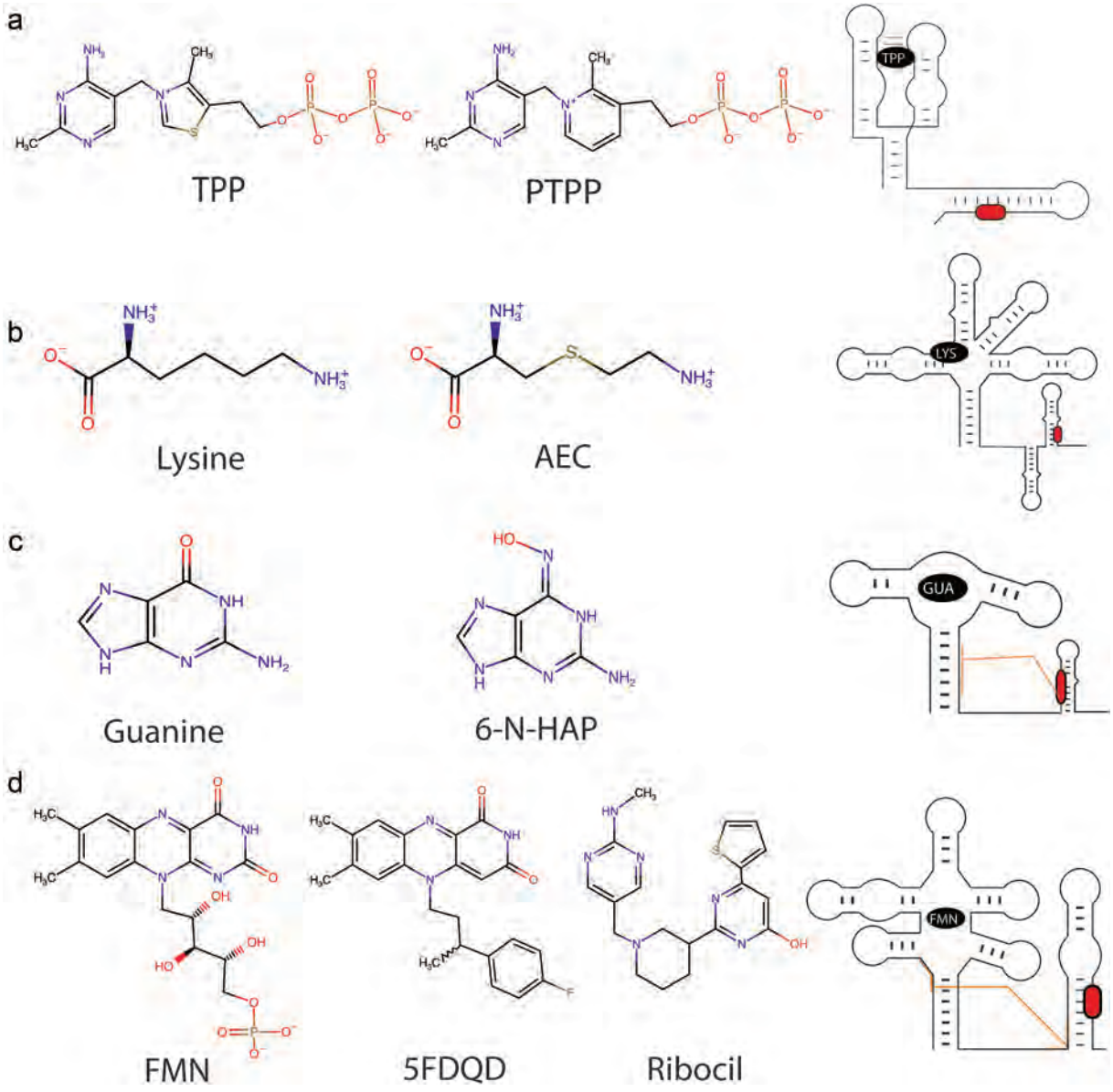

Figure 4. Chemical structures of native and synthetic ligands and their riboswitch binding. (a) the chemical structures of TPP, PTPP, and a structural schematic of the ligand bound TPP riboswitch. (b) the chemical structures of lysine, AEC, and a structural schematic of the ligand bound lysine riboswitch. (c) the chemical structures of guanine, 6-N-HAP, and a structural schematic of the ligand bound guanine riboswitch. (d) the chemical structures of FMN, 5FDQD, ribocil, and a structural schematic of the ligand bound FMN riboswitch.

their impact on gene expression using coupled *in vitro* transcription-translation assays. To this end, the luciferase gene was cloned downstream of the riboswitch, and the level of gene expression was measured following an incubation period with a given ligand analog concentration. For the readout, the luminescence of the sample was assessed upon the addition of a luciferase substrate. Notably, these analogs have demonstrated toxicity against some bacteria and fungi [31,32,48,49] (Figure 4(a)). However, bacterial resistance has emerged in the form of several mutations in the sequence of the TPP riboswitch, attesting to the speed with which such evolutionary adaptation can occur [31]. Thus, antibacterial approaches need to be developed that are less prone to trigger fast resistance.

Fragment-based drug screening has also been applied to identify potential compounds that can bind to the TPP riboswitch, but the fragments identified were unable to reduce the expression of downstream genes [31]. Other than ligand mimics, an engineered chimeric antisense oligonucleotide (ASO) was recently found to suppress pathogenic bacterial growth upon transport into the cell via the cell-penetrating peptide (CPP) pVEC. Specifically, the pVEC-ASO-1 binds to the TPP aptamer domain, causing the degradation of the embedding mRNA via RNase H cleavage [50]. The engineered ASO was shown to inhibit the growth of the food poisoning bacterium *Listeria monocytogenes* (*L. monocytogenes*), with no effect on *Escherichia coli* (*E. coli*) that lacks this TPP riboswitch [50]. Given that ASOs can rapidly be engineered based on sequence information alone, and will target a large region of sequence, they represent a novel class of 'information drugs' that may be harder to counter by bacterial mutations.

3.4. The lysine riboswitch

Another example that highlights the opportunity of riboswitches as antibiotic targets is the lysine riboswitch. This RNA motif controls transcription of the *LysC* genes, which encode aspartokinase II, the vital enzyme responsible for the first step in the metabolic biosynthesis of lysine, threonine, and methionine [51]. The binding of lysine stabilizes the transcription terminator, repressing transcription (Figure 4(b)). The previously discovered antibacterial compounds L-aminoethylcysteine (AEC) and DL-4-oxalysine bind to the lysC lysine riboswitch from *Bacillus subtilis* (*B. subtilis*, Figure 4(b)), leading to a decrease in transcription [51–53]. Soon mutations primarily in the aptamer domain of the *B. subtillis* and *E. coli* lysC riboswitches were discovered that cause resistance to AEC [54,55]. Later it was recognized that AEC also functions as amino acid substrate for lysyl-tRNA synthetase (LysRS) so that it becomes incorporated into proteins, leading to cytotoxicity, whereas mutations in the lysine riboswitch are responsible for acquiring resistance [56–58]. These findings indicate that off-target effects of riboswitch ligand mimics must not be overlooked, and that future antibiotics against the lysine riboswitch may need to be designed considering both targets for effective treatment [32,58]. By analyzing the crystal structures of the riboswitch, it was revealed that the ligand binding pocket has two openings situated at the C4 and N7 positions of the bound lysine, which can be used to expand the pharmacophore features for effective drug design [59,60].

3.5. The guanine riboswitch

The guanine riboswitch is a member of the purine sensing class and predominantly found in Gram-positive bacteria [61,62]. Four highly conserved guanine riboswitches in *B. subtilis* regulate vital genes including xpt-pbuX, pbuG, nupG, and the pur operon involved in purine transport, metabolism, and de novo purine biosynthesis [62,63]. When guanine is bound, the expression of the downstream genes is repressed [64]. There have been advances in antibiotic compounds targeting the guanine riboswitch, such as 6-N-hydroxylaminopurine (6-N-HAP) and 2,5,6-triaminopyrimidine-4-one (PC1), demonstrating its potential as a target [62,65] (Figure 4(c)). For example, in 2009 the Breaker group identified the antimicrobial guanine analog 6-N-HAP, which targets the *B. subtilis* xpt-pbuX guanine riboswitch [62] (Figure 4(c)). While the mechanism of action is unclear, upon 6-N-HAP binding the researchers observed a significant reduction in the expression of a reporter gene controlled by the xpt-pbuX guanine riboswitch. In 2010, the Johansson group discovered that 6-N-HAP also affects two *L. monocytogenes* guanine riboswitches that regulate genes lmo0573 and lmo1885, which code for a xanthine/uracil permease and a xanthine phosphoribosyl transferase, respectively [66]. They concluded that 6-N-HAP led to an increase in transcription termination of downstream genes. Intriguingly, 6-N-HAP not only impedes the expression of virulence factors but also boosts the bacterial mutation frequency by inducing an SOS response.

Another antimicrobial compound, the guanine analog PC1, identified by the Lafontaine group, selectively kills bacteria containing a guanine riboswitch that regulates the guaA gene coding for GMP synthetase in pathogenic *Staphylococcus aureus* (*S. aureus*) and *C. difficile* [65]. Similar to 6-N-HAP, validating the mode of action of PC1 remained challenging. Importantly, the authors showed that the selective antibacterial activity of PC1 in *S. aureus* does not cause toxicity in mice while reducing *S. aureus* infection in their mammary glands [65]. In addition, they suggested that resistance against PC1 is likely to be infrequent due to the essential nature of regulation of guaA by the guanine riboswitch. The potential of PC1 as an antibiotic was further demonstrated in 2013, when PC1 was used to treat cows infected with *S. aureus* [67]. PC1 showed an initial decrease in bacterial titer, which however was not maintained four weeks after treatment.

3.6. The FMN riboswitch

Rational drug design to develop synthetic ligands has also been applied to the FMN riboswitch. Biosynthesis and transport of riboflavin, which is a precursor of FMN, is carried out by a group of genes including ribD, ribE, ribA, ribH, and ypzK/ribT, known as the ribD operon [68,69]. The identification of roseoflavin, a chemical analog of FMN with antibiotic properties, led to the discovery of other riboflavin analogs with antibiotic potential, such as 5-(3-(4-fluorophenyl)butyl)-7,8-dimethylpyrido[3,4-b]quinoxaline-1,3(2 H,5 H)-dione (5FDQD) [68,70] (Figure 4(d)).

Independently, Merck discovered that the chemically unrelated small molecule ribocil also binds the ligand pocket of the FMN riboswitch but quickly triggers RNA mutations, leading the company to abandon this drug [45] (Figure 4(d)). More recently, new approaches using ASOs have begun to be applied [69], in particular to *S. aureus*, *L. monocytogenes*, and *E. coli* FMN riboswitches that utilize either the transcriptional or translational mechanisms of gene regulation (Figure 2). As for the TPP riboswitch, ASOs were designed to target the aptamer domain in the FMN riboswitch, common between the ribD operon and ypaA gene, leading to RNase H degradation of the corresponding mRNAs [69]. ASO was again delivered using the CPP pVEC, which passes through both prokaryotic and eukaryotic membranes and is not itself toxic. This work demonstrated that the ASO at a dose of 700 nM (4.5 µg/mL) inhibits bacterial growth to 80% and is not toxic to human cell lines [69].

Next, we will propose that targeting the interface of riboswitches and protein complexes is another mechanism that could be used to disrupt gene expression in pathogenic bacteria.

4. Riboswitches and their protein interfaces as potential drug targets

High-resolution structures of riboswitches showing their ligand binding pockets as well as their switching regions can be a boon for the development of new antibiotics. Indeed, the previous section showcased how ligand analogs mimic the presence of the essential ligand and derail bacterial gene expression [45,52,65,68,70–73]. However, this strategy has several caveats. First, high concentrations of these drugs are needed as they typically need to reach similar cellular levels as the actual ligand to trigger the riboswitch. Second, these antibiotics only target the RNA motif in isolation, precariously ignoring the critical interactions between the riboswitch and the gene expression machinery of the cell. In fact, essentially all cellular RNAs function as part of ribonucleoprotein complexes. Riboswitches are no exception in that they affect their own transcription and the translation of the downstream genes (Figures 1 and 2). Third, as the example of the lysC riboswitch demonstrates, small ligand mimics can easily have unintended off-target effects that make the clinical result of such compounds difficult to predict. Fourth, mutations in the riboswitch sequence can quickly confer resistance as found in multiple of the cases discussed above.

A larger molecule instead would overcome many of the drawbacks of small-molecule ligand mimics, as long as it can still cross the cellular membrane. For example, an antibiotic that bridges the riboswitch:protein interfaces involved in gene regulation could lower the risk of antibiotic escape and potentially have more predictability in a clinical context. Such antibiotics could either be developed with a broad range of targets because of conservation of the targeted RNAP and ribosome surfaces, or be made more narrowband if using specific riboswitch features not confined to the ligand binding pocket. That is, such antibacterials have the potential to achieve high species specificity and low microbiome toxicity

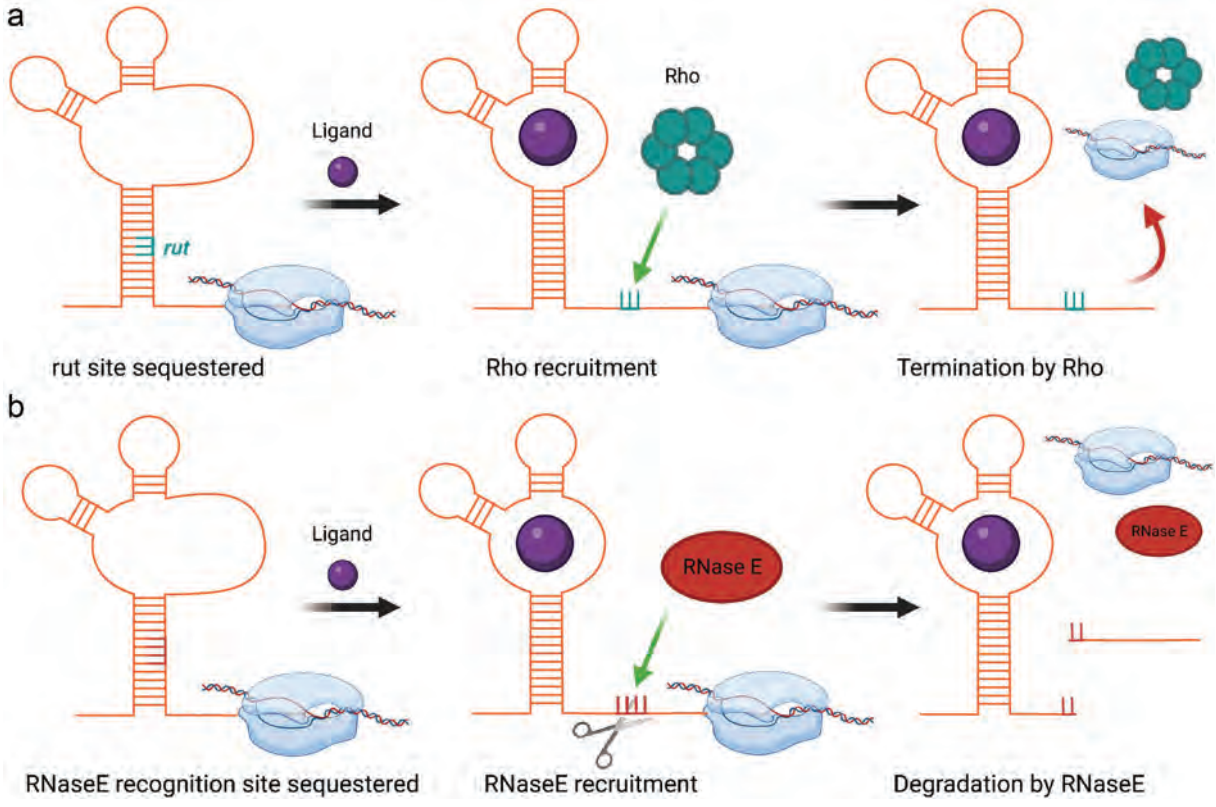

Figure 5. Additional mechanisms of riboswitch-mediated gene regulation involving Rho and RNase E. (a) in the ligand-free riboswitch, a rut site (blue) is sequestered but becomes exposed upon ligand binding, leading to termination by Rho factor which can dislodge the RNAP. (b) in the ligand-free riboswitch, an RNase E recognition site (red) is sequestered but becomes exposed upon ligand binding, leading to mRNA degradation by RNase E. Figure created with Biorender.

because riboswitches are evolved to operate under the specific cellular and environmental conditions of each bacterial species.

Recent studies have unveiled additional mechanisms of gene regulation that are mediated by riboswitches in response to their ligand and could be exploited for the design of more specific drugs targeting a specific bacterial phylum or species [74,75] (Figure 5). For instance, the TPP, FMN, and magnesium-sensing riboswitches have been found to trigger transcription termination in response to their respective ligands through the Rho transcription factor [76–78] (Figure 5(a)). Since this mechanism involves the sequestration or accessibility of the Rho binding site (i.e. Rho utilization site or rut sequence) as a function of riboswitch folding, it contrasts with polarity regulation in which the absence of translation of the nascent mRNA allows Rho-dependent transcription termination [30]. Bicyclomycin is a compound that specifically inhibits Rho activity [79,80], however, because of its low specificity toward a particular bacterial phylum, future avenues are needed that target Rho factor only in pathogenic bacteria and not in the commensal, beneficial species found in the human gut microbiome [81,82].

In another example of a so far understudied riboswitch mechanism, folding of the lysine and guanidinium-sensing riboswitches directly controls the accessibility of the mRNA degrading RNase E to its canonical recognition sequences, analogous to the rut sequence (Figure 5(b)). Interestingly, natural and synthetic compounds inhibiting RNase E activity have been identified [83] and one of them (glucosamine-6-phosphate) is also the natural ligand of the glmS riboswitch [84,85], suggesting that this particular riboswitch could be used to deliver such compounds.

Finally, the ligand-bound state of the fluoride-sensing riboswitch from *B. cereus* has been found to interfere with the activity of the essential transcription factor NusA through binding site sequestration [86]. As an essential transcription factor, NusA is important for the regulation of several steps of the transcription cycle [87], while also being involved in an 'immune system' within *E. coli* suppressing the activity of foreign genes [88] and favoring DNA repair [89], making this transcription factor an attractive target for the design of high-specificity antibiotics.

Taken together, because riboswitches fine-tune gene expression through terminating transcription, inhibiting translation initiation, as well as modulating essential protein cofactor recruitment (Figure 5), multiple mechanisms could potentially be exploited simultaneously through the design of high-specificity antibiotics targeting the mRNA-protein interfaces invoked by specific sets of genes (Figure 3(b)).

5. Conclusions

The risk of antibiotic resistance is a slow-motion public health crisis, as bacterial adaptation to the deployment of antibiotics will continue to result in a declining drug efficacy. Riboswitches provide a new avenue to explore for the development of antibacterials that may have few adverse effects on humans and animals, as they are primarily present in bacteria and offer unique mechanisms to target. Since many riboswitches are distributed across the high-priority pathogens identified by the WHO and CDC (Figure 1 and Table 1), anti-riboswitch drugs have the potential to address antibiotic resistance where it matters most [18]. Such antibacterials can function as ligand mimics that decrease critical gene expression as demonstrated for the guanine and lysine riboswitches. While this method has drawbacks, such as the tendency of riboswitch sequences to accumulate mutations that reduce drug binding affinity, there are other riboswitch targeting mechanisms that are less prone to mutational escape. New drug designs include ASOs that have been shown to decrease translation of TPP and FMN riboswitch containing mRNAs. Another promising mechanism may be to target the interface between riboswitches and protein complexes such as the ribosome, RNAP, or transcription factors. This approach may hold the advantage of reducing the likelihood of antibiotic escape, as well as enabling the design of antibiotics of high species specificity. In fact, while the large gene expression machineries are conserved across many bacterial species, riboswitches are often species-specific in sequence, allowing for the development of antibacterial agents that can be either broad- or narrowband. This is a significant advantage over many of the currently available small-molecule antibiotics, which often lack this level of specificity and can easily be overcome by bacterial resistance. More investment into new approaches is needed if we want to have a chance to win the arms race between antibiotic development and bacterial resistance.

6. Expert opinion

Since riboswitches are embedded near the 5′ end of mRNAs, they bind their respective ligand and change conformation while they are still being synthesized by RNAP [90], allowing for a dynamic response to a bacterial cell's physiological conditions. Due to their narrow temporal window for gene regulation, riboswitches have the ability to interact with the nearby RNAP and its accessory proteins, as well as the pioneering ribosome, providing an underexplored mechanism to be exploited for the design of antibiotics. As a gateway for the regulation of transcription processivity, the positively charged RNA exit channel of RNAP constitutes an attractive platform in which nascent transcripts could establish key contact points to regulate the efficiency of RNA synthesis. Within the RNA exit channel, subdomains such as the ß-Flap or the Zinc Binding Domain (ZBD) have been found to participate in numerous regulatory pathways during the transcription cycle [91–96]. Even though the core RNAP structure is very well conserved in all domains of life, particular residues have been found only in bacteria [97] and could constitute a specific interface targeted by specific riboswitch features as well as future antibiotics. That is, going beyond directly targeting the ligand binding pocket, exploiting riboswitch interactions with the gene expression machinery presents a potentially fertile ground for novel drug design.

Recent work on a preQ$_1$-sensing transcriptional riboswitch from *B. subtilis* identified specific interactions between RNA and RNAP involved in riboswitch function [98]. In this work,

the Walter group characterized the riboswitch folding dynamics in the presence of both RNAP and DNA template. The authors found that RNA polymerase pauses just downstream of the riboswitch aptamer so that the proximity of RNAP profoundly affects riboswitch folding. Conversely, the ligand-free RNA nestled into the exit channel extends RNAP pausing; only upon ligand binding is the pause released. More recently, determination of the cryo-electron microscopy (cryo-EM) structure of the paused elongation complex, combined with Molecular Dynamic Flexible Fitting (MDFF), revealed the specific RNA-RNAP interactions responsible for pausing and unpausing [99]. Notably, many of the close contacts between riboswitch and RNAP involve protein domains conserved across all domains of life and/or within the bacterial phylum, however, others could be species specific, giving rise to opportunities for either broad- or narrowband antibacterial compounds.

Another mechanism to potentially be exploited for drug design is invoked by translational riboswitches in the form of transcription-translation coupling into the pioneering expressome complex [100]. In bacteria, because of the lack of cellular compartmentalization RNAP is closely followed by the pioneering ribosome, allowing the translation of an mRNA into protein concomitantly with RNA synthesis [101]. While still in debate for some bacterial species [102], this process involves a physical contact between elongating RNAP and ribosome [103–105]. Disturbing this RNAP-ribosome interface could lead to disruption of the coupling and premature transcription termination [30] or mRNA degradation [106], and ultimately bacterial cell death.

In recent work, a preQ$_1$-sensing translational riboswitch from *B. anthracis* was found to modulate transcription-translation coupling upon ligand binding by altering the likelihood of ribosome recruitment [107]. By surveying riboswitch transcription in real-time, the Walter group found that the ribosome positively promotes transcription elongation in the absence of ligand. In contrast, the presence of cognate ligand led to a similar RNAP transcription rate with and without ribosome, suggesting that translation initiation in the wake of RNAP is controlled by ligand binding to the riboswitch. These findings open the door to the design of new antibiotics targeting the ribosome-RNAP, riboswitch-ribosome, riboswitch-RNAP or riboswitch-RNAP-ribosome interfaces.

Taken together, the many avenues offered by riboswitch-directed drug design may herald a new golden age for antibiotics, which would be much needed in the face of largely unmitigated bacterial drug resistance.

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Declaration of interest

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