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Review

Riboswitches: From living biosensors to novel targets of antibiotics

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ABSTRACT

Riboswitches are generally located in 5′-UTR region of mRNAs and specifically bind small ligands. Following ligand binding, gene expression is controlled mostly by transcription termination, translation inhibition or mRNA degradation processes. More than 30 classes of known riboswitches have been identified by now. Most riboswitches consist of an aptamer domain and an expression platform. The aptamer domain of each class of riboswitch is a conserved structure and stabilizes specific structures of the expression platforms through binding to specific compounds. In this review, we are highlighting most aspects of riboswitch research including the novel riboswitch discoveries, routine methods for discovering and investigating riboswitches along with newly discovered classes and mechanistic principles of riboswitch-mediated gene expression control. Moreover, we will give an overview about the potential of riboswitches as therapeutic targets for antibiotic design and also their utilization as biosensors for molecular detection.

Contents

Abbreviation: 5′UTR, 5′ Untranslated region; miRNA, microRNAs; siRNA, small interfering RNA; c-di-GMP, cyclic di guanosine monophosphate; c-di-AMP, cyclic di adenosine monophosphate; SD, Shine–Dalgarno; MDR, multiple drug resistance; BLISS, the Breaker Lab Intergenic Sequence Server; PAGE, Polyacrylamide Gel Electrophoresis; CM, Covariance Model; *glmS*, glucosamine-6-phosphate; ZMP, 5-Amino-4-imidazolecarboxamide riboside 5′-monophosphate; GFP, green fluorescent protein; 2,4 DNT, 2,4-dinitrotoluene.

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1. Introduction

There are several control mechanisms to modulate the expression of genes in response to numerous cellular conditions. The combination of protein signaling and factors which control transcription, translation and messenger RNA (mRNA) processing or degradation is the most common known mechanism of gene expression regulation. Furthermore, there is a developing idea that RNA has a definite role in posttranscriptional control mechanisms. *Trans*-acting riboregulators such as microRNAs (miRNA) and short interfering RNAs (siRNA) regulate gene expression by forming base paired structures with target mRNA and through various protein mediated pathways. On the other hand, *cis*-acting regulating RNAs are usually located in non-coding parts of mRNA (reviewed in Waters and Storz, 2009).

RNA based regulation provides several advantages. First, since mRNA is the direct target, a rapid response is ensured. Second, for small RNAs (sRNAs) acting through binding to mRNAs, specific and fast recognition can be gained with a low number of base pairings (Aiba, 2007; Vogel, 2009). Third, specificity may evolve more rapidly since one nucleotide change can be sufficient to change specificity (Mandal and Breaker, 2004a). Finally, the capacity of RNAs to modulate their conformation upon binding increases the number of contacts with a given ligand or makes the recognition of multiple targets easier.

Riboswitches are genetic regulatory elements usually found in the 5′-UTR (untranslated regions) of mRNA (Gelfand et al., 1999; Henkin, 2000; Mandal and Breaker, 2004b; Nudler and Mironov, 2004) (Fig. 1A) and are widely distributed in bacteria (reviewed in Winkler and Breaker, 2005). The term riboswitch is greatly associated with binding to specific small molecules without protein factors involvement and eventually controlling gene expression by allosterically altering dimensional structures of the mRNA. They are mostly located in the upstream regions of bacterial mRNAs that encode biosynthetic enzymes or metabolite transporters. These sequences comprise of two parts: aptamer domain and expression platform. Generally, ligand binding to the aptamer domain of a riboswitch stabilizes specific structural elements of joining expression platform, which modulates the expression of downstream genes. As a result, they regulate the process of transcription and/or translation (Fig. 1B).

Riboswitches can bind and sense cellular metabolites such as amino acids and derivatives [lysine, glycine, *S*-adenosyl methionine (SAM), *S*-adenosyl homocysteine (SAH)], carbohydrates [glucosamine-6-phosphate (Glcn6P)], coenzymes [flavin mononucleotide (FMN), thiamin pyrophosphate (TPP), coenzyme B_{12}], nucleobases and their derivatives [adenine, guanine, c-di-GMP, c-di-AMP, pre-queuosine (preQ1)] (Barrick and Breaker, 2007a; Sudarsan et al., 2008) and ions like Mg^{2+} (Cromie et al., 2006; Dann et al., 2007). Some of the recent discovered riboswitches are c-AMP-GMP (Nelson et al., 2015; Kellenberger et al., 2015a), 5-Amino-4-imidazolecarboxamide riboside 5′-monophosphate (ZMP) (Kim et al., 2015), cations Mn^{2+} (Dambach et al., 2015; Price et al., 2015) and Ni^{2+}/Co^{2+} (Furukawa et al., 2015) binding motifs and anions such as fluoride (Baker et al., 2012). The particular interest about anion binding to RNA is how a negatively charged RNA could bind to a negatively charged ligand. Ren and colleagues found out that bound fluoride could be encapsulated with three Mg^{2+} ions which are coordinated to water molecules and RNA backbone phosphates (Ren et al., 2012).

Gene regulation mechanisms by riboswitches are in various levels of transcription and translation (Waters and Storz, 2009). The first mechanism involves the ligand-dependent formation of a Rho-independent termination which is a GC-rich stem followed by a poly-U tail. This structure, *e.g.* SAM riboswitches, can inhibit RNA polymerase and stop transcription elongation. In the second mechanism such as SMK-box in *metK* gene, riboswitches decrease the ribosome access to the Shine–Dalgarno (SD) or AUG start codon sequences in a stem-loop formation. Furthermore, as an exceptional mechanism, *glmS* riboswitch perform self-cleavage after binding to glucosamine-6-phosphate, leading to mRNA degradation (Collins et al., 2007). Additionally, there are combined forms of riboswitch dependent gene regulation such as a tandem riboswitch (Mandal et al., 2004) or two different riboswitches adjacent to each other (Spinelli et al., 2008; Sudarsan et al., 2006).

Riboswitch ability to function as a specific and selective sensor for intracellular metabolites in a protein-independent manner makes it a promising research area to develop new types of engineered aptamers and biosensors. On the other hand, riboswitches have important roles in the regulation of vital pathways in the cell through feedback mechanisms. As a result, they have been considered theoretically and practically as potential new targets for antibiotic design in order to find a solution for multiple drug resistance (MDR) (Blount and Breaker,

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2006). In the following review we aimed to review different aspects of riboswitches nature and particularly their characteristics as ligand binding sensors and therapeutic tools.

2. History of riboswitches

It was known for a long time that metabolites synthesis or transportation in the cell is regulated by regulating proteins. Besides, regulatory RNAs which cooperate with proteins were introduced many years ago. Discovering of ribozymes in 1980s (Kruger et al., 1982) as well as using directed evolution strategies in 1990s to make aptamers (Ellington and Szostak, 1990) supported the speculation that RNA molecules function both as information storage systems and as the biochemically active biomolecules (Joyce, 1991). Gradually, it was speculated that natural RNA aptamers making RNA switches could have been existed in the organisms. The clue was to start from some metabolites with unknown regulation mechanisms.

In one of the first attempts, Grundy and Henkin showed a conserved sequence in the upstream of S-box gene family involved in the biosynthesis of methionine and cysteine (Grundy and Henkin, 1998). Based on the computational prediction, this conserved sequence can form a secondary or tertiary structure. Experimentally, it was demonstrated that the conformation of this structure altered when it bound to a regulatory factor. Other regulating "boxes" were also determined in upstream of mRNAs such as THI box (TPP regulating) by Rios et al. (Miranda-Rios et al., 2001) and RFN box by Gelfand et al. (Gelfand et al., 1999). On the other hand, because no protein regulators for the metabolism of thiamine (B_1), riboflavin (B_2) and cobalamin (B_{12}) vitamins were found (Miranda-Rios et al., 2001; Nou and Kadner, 1998), regards pointed to the mentioned conserved sequences called "boxes" in the upstream of mRNAs (Gelfand et al., 1999; Stormo and Ji, 2001). The term "riboswitch" was first coined by Breaker and co-workers (Nahvi et al., 2002), confirming the existence of a 5′-UTR sequence in the *Escherichia coli btuB* mRNA which selectively binds to the coenzyme B_{12}. It was shown that this interaction exerts its effect without the interpretation of proteins. Also, they developed "In-line probing" method as a useful approach to detect the conformational changes of mRNA due to ligand binding. Afterwards, other non-coding mRNA structures which directly bind to FMN (Mironov et al., 2002) and TPP (Mironov et al., 2002; Winkler et al., 2002a) were also determined. Accordingly, the significance of this discovery in the probable evolutionary origin of riboswitches was declared. Meanwhile, the pattern of "two-part structure" in the riboswitches including "aptamer domain" and "expression platform" as well as typical regulation mechanisms of "transcription termination" and "translation attenuation" were established.

With the similar approach other riboswitch classes like purines (Mandal et al., 2003), *S*-adenosylmethionine (SAM) (Epshtein et al., 2003; McDaniel et al., 2003; Winkler et al., 2003), lysine (Grundy et al., 2003) and molybdenum cofactor (Regulski et al., 2008) (Moco) were identified and characterized. However, finding new riboswitches has definitely been a challenging area in the past years. Using computational methods, in 2007 and 2010, Weinberg and colleagues introduced several motifs supposed to be riboswitches and called them "orphan riboswitches" (Weinberg et al., 2007; Weinberg et al., 2010) which means their natural ligands were unidentified. Although limited number of them was identified in the past years, most of the ligands of orphan riboswitches are still unknown. Some possible reasons are presented in the following. First, it is possible that the ligand itself is

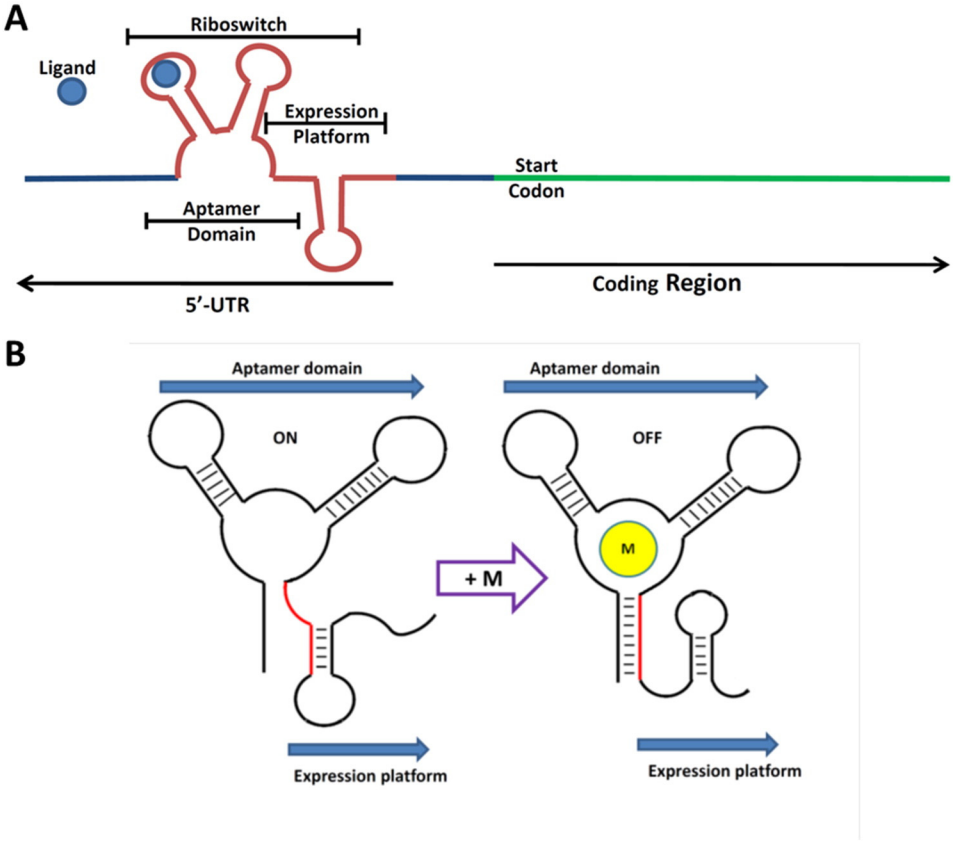

Fig. 1. Riboswitches schematic structures (A) Schematic structure of typical mRNA consisting of 5′-UTR and coding region. Red part is the riboswitch part consisting of aptamer domain and expression platform. The ligand binds to the structurally conserved aptamer domain of the riboswitches and consequently changed the conformation of expression platform in a way to stop transcription and/or translation of coding region. (B) Schematic of riboswitch-mediated repression of transcription. This is one example of changing the conformation of expression platform after binding of the ligand (M). Here an anti-terminator stem-loop structure can alter to a terminator stem-loop which halts transcription process. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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unknown for scientists as for c-di-AMP riboswitches (Ren and Patel, 2014). Besides, some ligands might have not been important enough for scientists to test like fluoride (Ren et al., 2012) or ZTP (Kim et al., 2015). It is also possible that the regulation mechanism of the ligand was not completely known as for Mn^{2+} riboswitches (Dambach et al., 2015). However, new screening methods as well as examining several libraries led to the discovery of some riboswitches recently. Some of the methods will be discussed in the following parts.

3. Mechanisms of riboswitches

Basically, ligand binding to the aptamer domain of riboswitches regulates the downstream gene or genes (Mandal and Breaker, 2004b). In addition, RNA folding variations influence various processes that contribute to the gene expression efficiency. Consequently, there are different types of gene regulation mechanisms used by riboswitches (Fig. 2).

One of the most general mechanisms is the modulation of transcription termination (Barrick and Breaker, 2007a). Transcription terminator, a stem trailed by a chain of uridine residues (Yarnell and Roberts, 1999; Gusarov and Nudler, 1999), stops RNA polymerase and consequent transcription (Fig. 2A). Conformation of aptamer domain through metabolite binding controls the formation of the terminator by making a competitive anti-terminator.

In the other common mechanism, similar structures are used by riboswitches to control ribosome access to the ribosome binding site or Shine-Dalgarno (SD) sequence, thereby halting translation initiation (Fig. 2B). Third mechanism is known specifically for eukaryotic riboswitches. TPP riboswitch is the only riboswitch class (Mironov et al., 2002; Winkler et al., 2002a) discovered in plants and fungi (Sudarsan et al., 2003). It was demonstrated that in the eukaryotes this riboswitch controls splicing of mRNA (Wachter et al., 2007; Cheah et al., 2007; Croft et al., 2007; Kubodera et al., 2003). There are also some suggestions about the contribution of protein factors, such as transcription termination factor Rho and RNases (Hollands et al., 2012; Caron et al., 2012). In this type of mechanism, riboswitches contain anti-SD sequences and both transcriptional and translational regulation levels are performed (Breaker, 2012). In another mechanism for SAM-I riboswitch (Rodionov et al., 2004), following the alteration of sulfur concentration an antisense RNA is produced (Andre et al., 2008). However, the true mechanism may not be completely revealed.

There is usually a switching sequence located between the aptamer domain and the expression platform. This sequence element could just be integrated into the structure of one of the two domains (Winkler and Breaker, 2005; Grundy and Henkin, 2006). Changing the structure of switching domain leads to the incorporation of this element into the expression platform and the formation of the competitive structure in the expression platform is prevented (Nudler and Mironov, 2004; Winkler and Breaker, 2005; Winkler, 2005; Winkler and Breaker, 2003). Hence, the switching sequence is a communication link between two domains and has an essential role in the regulation output.

In a very recent publication, a protein involvement was recognized for FMN riboswitch activity as a dual control mechanism for riboflavin in *Bacillus subtilis*. In summary, protein RibR as a superordinate regulator, counteracts FMN riboswitch in *B. subtilis*, allowing this bacterium to fine-tune riboflavin metabolism. The possible reason for this dual metabolic control is declared as coupling of sulfur and riboflavin metabolism (Pedrolli et al., 2015).

Considering the common mechanisms of riboswitch-based regulation, there are two functional states for riboswitches: the ligand bound and ligand-free states. However, some recent studies proposed three or more stable conformations during riboswitch function (Furtig et al., 2015). For example, adenine-sensing riboswitch in *Vibrio vulnificus* showed three distinct stable conformations. This kind of switching can play a key role in the bacteria adapting to environments with varying temperatures (Reining et al., 2013).

Another intriguing gene control mechanism of riboswitches is mostly known for *glmS* riboswitch/ribozyme explained in the following section.

3.1. glmS riboswitch/ribozyme

The representatives of this riboswitch class bind to glucosamine-6-phosphate (GlcN6P) which causes self-cleavage of related mRNA encoding protein enzyme glucosamine-6-phosphate (GlcN6P) synthetase (GlmS) (Winkler et al., 2004). The self-cleavage produced fragment bears a 5′-OH group which makes it a degradable target for RNase J1. The self-cleavage and nuclease-mediated degradation finally result in reduced gene expression (Collins et al., 2007). In addition to *glmS* riboswitch's mechanism of action, its structural rigidity makes it an atypical riboswitch. The *glmS* riboswitch is a rigid and completely assembled structure before GlcN6P binding. According to different analog studies, three functional groups of GlcN6P including the anomeric hydroxyl, the amine and the phosphate are important to interact with *glmS* riboswitch/ribozyme. As a result, some compounds such as glucosamine (GlcN), L-serine, serinol, Tris and ethanolamine which present vicinal amine and hydroxyl groups are weak activators of *glmS* riboswitch/ribozyme (McCarthy et al., 2005).

GlcN6P synthetase catalyzes the first step in the metabolic pathway of the bacterial cell wall synthesis. Therefore, it has been suggested that *glmS* riboswitch/ribozyme could be a valuable target to design novel

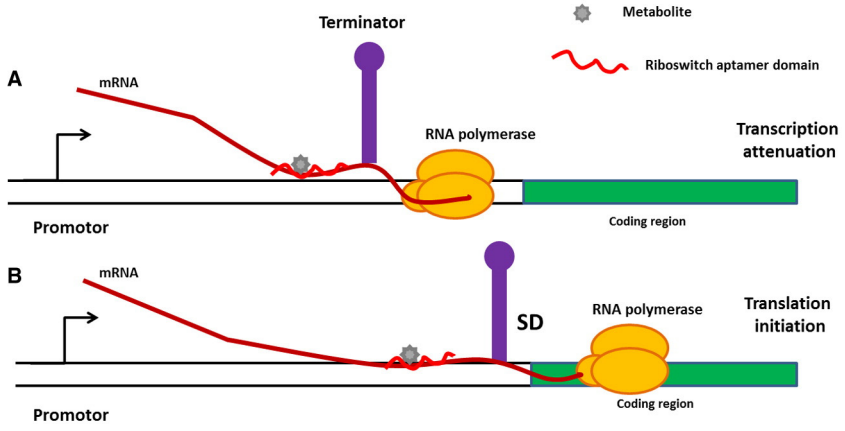

Fig. 2. Riboswitch gene control mechanisms (A) Regulation of transcription attenuation: In this mechanism RNA polymerase action stop by formation of a terminator stem-loop structure following to the metabolite binding. Once the ligand isolate from the riboswitch, anti-terminator structure is formed again and transcription starts over. (B) Regulation of translation initiation: In this mechanism binding of the ligand causes Shine-Dalgarno (SD) sequence masking by forming a stem-loop structure. Consequently, ribosomes cannot continue translation. SD masking will be removed after ligand separation.

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antibacterial compounds leading to bacterial cell wall damage (Ferre-D'Amare, 2010). Additionally, because of rigid catalytic core structure and abundant number of representatives in pathogenic bacteria, the *glmS* ribozyme is a theoretically suitable antibiotic target. However, it is worth to mention other RNA regulating mechanism of GlmS protein production through sRNAs. Two main sRNAs playing key role in *glmS* gene regulation are GlmZ and GlmY. GlmZ sRNA directly binds to *glmS* transcript which releases the RBS of these transcripts and enhances the production of GlmS protein. On the other hand, GlmY sRNA controls GlmZ processing and inhibits its inactivation (Urban and Vogel, 2008). Considering these alternative mechanism of *glmS* regulation, *glmS* riboswitch inhibition doesn't seem adequate mechanism of an antibiotic activity. Studies regarding the development of antibiotics targeting *glmS* riboswitch/ribozyme are more discussed in section "development of riboswitch-targeted antibiotics".

4. Methods for riboswitch discovery

Riboswitches, which structurally act as receptors for specific ligands, are located in the non-coding segments of mRNAs in many bacteria. Aptamer domains of riboswitches are conserved sequences and compose precise three-dimensional structures selectively bind a target molecule. The sizes of known aptamers vary from 35 to around 200 nucleotides in length. However, expression platforms vary broadly in size, nucleotide sequence and structural pattern (Winkler and Breaker, 2005). In addition, riboswitches are capable of discriminating the main ligands from similar effectors with apparent dissociation constant (K_D) values ranging from picomolar to low micromolar concentrations (Mandal and Breaker, 2004b).

Basically, the process of finding riboswitches, whether the novel or common types, includes three steps: identification, determination and confirmation (Regulski and Breaker, 2008). Each step has its own intricacy and challenges.

4.1. In silico approaches

Currently, in order to identify new riboswitch class candidates, comparative genomic approaches such as BLAST are applied (Barrick et al., 2004; Corbino et al., 2005). More professionally, the Breaker Lab Intergenic Sequence Server (BLISS), as a comprehensive database, provides records of homologous RNAs in intergenic regions identified before *via* BLAST (Barrick et al., 2004; Blount et al., 2015). Moreover, secondary structure models are then predicted for promising RNA motifs. However, several criteria are employed in order to reject non-riboswitch motifs including: (1) failing to show features of structured RNAs- when the predicted structures of sequence homologs are poorly conserved, (2) showing repetitive elements which means elements present several times in the genome with high sequence conservation, but little structure conservation, (3) presence of protein-binding motifs' features such as consisting of a single or tandem small hairpins architecture to bind homodimeric proteins.

Generally, as Breaker group described (Weinberg et al., 2007; Weinberg et al., 2010), selecting an RNA motif as a riboswitch candidate is based on assessment of sequence and structure conservation, covariation and gene context. Covariation involves in the mutation of Watson-Crick or G-U base pair in a way that the secondary structure is protected. According to the degree of conservation and covariation, an RNA motif is established as a riboswitch candidate or not. All these data are refined and expanded by further BLAST searches. By means of mentioned computational approaches it is expected that more regulation sequences, particularly riboswitches, are going to be figured out.

Because of high degree of general structures in riboswitches and the conservation of key nucleotides in aptamer domains (often exposed in bulges and loops) and the phenomenon of co-variation in order to maintain secondary structures, *in silico* approaches have remarkable role in suggesting novel riboswitch candidates (Weinberg et al., 2007; Barrick et al., 2004; Kazanov et al., 2007). Several servers and programs were developed in order to predict riboswitches in the sequences such as Denison Riboswitch Detector (DRD) (Havill et al., 2014), RiboSW (Chang et al., 2009), Riboswitch Explorer (Abreu-Goodger and Merino, 2005), Riboswitch finder (Bengert and Dandekar, 2004), Riboswitch Scanner (Mukherjee and Sengupta, 2015). Based on the most powerful tool, Covariance Model (CM), using comparative genomics, potential riboswitches have been predicted and introduced, including *glmS*, *gcvT*, *ykoK*, *ydaO/yuaA*, *ykkC/yxkD*, *yybp/ykoY*, *yjdf* and *pfl* (Weinberg et al., 2007; Weinberg et al., 2010; Barrick et al., 2004). They were called "orphan riboswitches" whose ligands might be recognized by extra biochemical and genetic experimentation in the future. Increasingly, targets of some of these motifs have been confirmed experimentally, supporting the validity of bioinformatics tools in this area. For example, the *ykoK* motif detects Mg^{2+} level, the *glmS* riboswitch senses the glucosamine-6-phosphate (Winkler et al., 2004; Klein and Ferre-D'Amare, 2006; Cochrane et al., 2009), the *yybp/ykoY* motif binds Mn^{2+} (Dambach et al., 2015; Price et al., 2015) and the *ydaO* motif binds c-di-AMP (Ren and Patel, 2014; Gao and Serganov, 2014). Some other previously orphan riboswitches have been assigned just recently with ligands such as ZMP for *pfl* motif (Kim et al., 2015) and cations Co^{2+}/Ni^{2+} for *czc* motif (Furukawa et al., 2015).

4.2. Biochemical verification of riboswitch-ligand binding

Having identified a promising riboswitch candidate, it is critical to show that it is a definite genetic control element (determination) which specifically binds a metabolite beyond protein interference (confirmation). To find out the specific ligand, gene association is one of the best indicators of ligand specificity for riboswitches, as usually downstream genes are regulated by the riboswitch specific ligand in metabolic pathways. Unfortunately, rare riboswitches and even some common riboswitches candidates do not provide any gene association to find a clue concerning ligand identification, especially riboswitches related to unknown function genes. This issue was also true about the latest ligand identification on *yjdf* motif (Li et al., 2016). As a result, Li and colleagues used a strategy of testing various chemical compounds, showing that *yjdF* RNAs can regulate gene expression by binding to a large diversity of azaaromatics. However, the natural ligand for "azaaromatics riboswitches" has still remained unknown.

Determination and confirmation of direct binding of a ligand to riboswitch aptamers can be completed by using a variety of techniques. Some methods have the potential to analyze the nucleotides that are involved in ligand binding, measurement of apparent K_D values as well as characterizing the ligand-induced RNA conformational change. Two such methods are called as In-line probing and Selective 2′-Hydroxyl Acylation Analyzed by Primer Extension (SHAPE).

In-line probing assays have been used widely to clarify the secondary structure and binding characteristics of riboswitch aptamer domains. It is known that RNAs, based on their structure, are naturally able to degrade differentially (Soukup and Breaker, 1999). The main RNA degradation mechanism is an 'In-line' nucleophilic attack by the 2′ oxygen on the neighboring phosphorus center. Levels of this kind of degradation are based on the folding structure of RNA (Soukup et al., 2001). Accordingly, in the case of riboswitches, after running the labeled RNA in Polyacrylamide Gel Electrophoresis (PAGE), metabolite treated and free state patterns of RNA cleavage would be demonstrated. Further analysis of these differences leads to more information about the secondary structure properties of the RNA motif.

Selective 2′-Hydroxyl Acylation Analyzed by Primer Extension (SHAPE) has been employed on riboswitch aptamers with similar results to the outcomes of In-line probing assays. In this method, structure-dependent alterations happen following the reaction of 2′-hydroxyl groups to chemical agents such as *N*-Methylisatoic anhydrideare. These changes are applied to evaluate the modifications by reverse transcription of chemically treated RNA templates. Following to the ligand

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binding and consequent changes in RNA structure, transcription is blocked at the sites of modification. Similar to In-line probing, provided information can be used to understand and develop precise models of RNA secondary structures and conformational changes due to ligand binding (Wakeman and Winkler, 2009; Warner et al., 2014).

In addition to mentioned chemical probing methods, there are some methods for studying ligand binding of riboswitch aptamer regardless of its conformational changes.

Equilibrium dialysis is a suitable method to study the riboswitch-ligand affinity if a radiolabeled ligand is available (Poiata et al., 2009). It is based on the distribution of radiolabeled ligand between two-chambered equilibrium dialysis systems. The distribution will shift to the chamber containing the aptamer RNA. However, the distribution will be equal if a mutant RNA or a competitive ligand is present.

Isothermal Titration Calorimetry (ITC) is another method to determine the binding affinity and stoichiometry as well as detailed thermodynamic parameters in riboswitch-ligand interactions. Binding of different riboswitches to their cognate ligands can vary from high endothermic to strongly exothermic. Due to the thermodynamic dependency of these specific interactions to parameters such as temperature, pH, Mg^{2+} concentration and ionic strength, comparing of the results from different laboratories need to be done more cautiously (reviewed in Zhang et al., 2014).

Surface plasmon resonance (SPR) analysis is another main technique for quantification of affinity, binding enthalpy and binding kinetics of macromolecular interactions. It has the advantages of real-time investigation on label-free biomolecules' interactions. As a result, SPR has been used to study some of the riboswitch-ligand interactions in the past years (Schaffer et al., 2014; Mayer et al., 2007; Jenkins et al., 2011).

4.3. Genetic validation of riboswitch-mediated gene regulation

In order to study gene control mechanisms used by riboswitches, the desired RNA motif is fused to a reporter construct such as *lacZ* or GFP as a reliable method to determine whether an RNA motif is a gene expression regulator in bacteria or not (Block et al., 2010; Mulhbacher et al., 2010). In this method, after providing the construct, medium is supplemented with the desired ligands (usually supplemented in minimal medium). Then, the effect on the gene expression is examined by assessing the production of the reporter gene *e.g. lacZ* in the presence and absence of the ligand. Besides, it is also possible to discover the gene regulation mechanism by performing some site-directed mutations in both aptamer domain and expression platform sequences and measure the production of reporter gene in response to the ligand.

4.4. Finding riboswitches using genome-wide mapping of transcriptional termination

Dar and colleagues recently developed an experimental approach for high-throughput genome-wide mapping of conditional transcriptional termination called term-seq (Dar et al., 2016). They basically applied quantitative RNA sequencing on the genome of individual bacteria as well as microbiomes to identify the ribo-regulators of specific genes. Accordingly, the method was employed in order to detect the influenced regulators in response to given metabolites. Using term-seq it is possible to overcome the difficulties of finding riboswitches based on comparative genomics. Therefore, known riboswitches like lysine riboswitch was confirmed with this method and new regulators were identified in the genome of *B. subtilis*, *Listeria monocytogenes* and *Enterococcus faecalis*. In addition, treating the resistant bacteria with sublethal concentrations of some antibiotics, new regulators involved in antibiotic resistance were detected which will be discussed in the Section 6.1. Although this method is capable of finding metabolite-responsive regulators, it is limited to differentiate between riboswitches, attenuators and protein-dependent termination. However, this novel methodology can accelerate the process of finding new riboswitches as well as assigning function to unknown ribo-regulators.

5. Engineered riboswitches

5.1. Background

Conformation switching of riboswitches allows us to employ them to design new genetic switches using different nucleic acid engineering methods. Moreover, modification of an aptamer domain can be used to create a new construct as a non-immunogenic genetic control element. For instance, the aptamer domain is joined to other non-natural aptamers and generates a new recognition domain with desired effector compounds. As an important example, a recent study demonstrates an artificial riboswitch, a ligand-dependent self-cleaving ribozyme (aptazyme), which can reduce the expression of an RNA virus structural gene. This was one of the first attempts of using riboswitches for replication control of human-pathogenic viruses and shows the strength of riboswitches in applied biology (Ketzer et al., 2014). In addition, RNA switches can be very effective tools for manipulating the bacteria in order to respond pollutants or disease markers (reviewed in Hartig, 2007).

On the other hand, generally speaking, "biosensors" are detecting tools which bind ligands by a recognition element leading to a detectable signal production such as fluorescence or electrochemical signal (Wang et al., 2011; Ilkhani et al., 2011; Pournaghi-Azar et al., 2010; Pournaghi-Azar et al., 2006). Before the discovery of natural metabolite binding RNAs (riboswitches), their artificial kind, RNA aptamers, had been developed through *in vitro* selection (Wilson and Szostak, 1999; Edwards et al., 2007). Aptamers are eminent tools to be applied as sensors for their binding molecules. They are capable of chemical modifications to insert signal producing moieties or attach to different surfaces. As a result, notable range of aptamer-based *in vitro* biosensors can be designed (Sefah et al., 2009; Navani and Li, 2006). However, molecular detection characteristics of riboswitches make them promising candidates for production of biological sensors. Moreover, it is more feasible to create an artificial riboswitch which senses a desired molecule by modifying an existing riboswitch.

5.2. Riboswitch engineering

In order to generate a special riboswitch responding to a selective molecule, two goals should be fulfilled including an RNA aptamer that distinctively binds the ligand and a linking part which controls gene expression. However, due to the complexity of aptamer domains, designing a completely novel riboswitch is a challenging task. Consequently, engineered riboswitches have been generated from established RNA aptamers; either created *in vitro* or from natural riboswitches. A range of approaches for designing, screening and selection should be used to recognize elements where aptamer binding results in genetic regulation.

5.2.1. Selection methods

One of the strategies for selection is based on providing a selective growth media to select clones harboring desired construct. In a study, this method was employed using *tetA* tetracycline resistance gene which also sensitizes cells to nickel (Nomura and Yokobayashi, 2007a). Using this method several synthetic riboswitches were isolated (Sharma et al., 2008; Nomura and Yokobayashi, 2007b). Another strategy to recognize desired riboswitches in the libraries is to use reporter proteins as detectable signals to quantify the expression level of preferred genes. In the large libraries, high throughput screening methods assess reporter expression in different clones. In one of the first attempts, using a developed high-throughput screening, new riboswitches were identified which trigger β-galactosidase expression in *E. coli* (Lynch et al., 2007). Afterwards, a screen based on

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fluorescence-activated cell sorting (FACS) in larger libraries was developed which identified theophylline responsive synthetic riboswitches (Lynch and Gallivan, 2009). In addition, combining expression of green fluorescent protein (GFP) with FACS was used to search a library of potential active riboswitches (Fowler et al., 2008). The next approach to recognize appropriate clones was based on the changes in cell motility. CheZ protein has a great role in the migration of *E. coli*. By insertion of *cheZ* gene in the downstream of theophylline binding riboswitches, cell migration on semi-solid agar revealed the desired colonies (Topp and Gallivan, 2008).

5.2.2. Artificial riboswitches and applications

One of the first artificial prokaryotic riboswitches was produced in 2004 (Suess et al., 2004) based on a rational design similar to natural mechanism. In this study, after attaining structural information of theophylline binding aptamer, an artificial riboswitch was designed. This construct worked at the level of translational initiation as theophylline binding close to aptamer causes structural change in the inhibitory helix and increases the expression. Most of the engineered riboswitches are prepared using mostly theophylline or tetracycline aptamers (reviewed in Wittmann and Suess, 2012; Berens and Suess, 2015) and a few neomycin aptamers (Weigand et al., 2008). Therefore, it seems necessary to develop new aptamer domains as well as the screening methods in order to enhance the range of engineered riboswitches' applications. The ligands should be active *in vitro* as well as *in vivo*. Some of the attempts using re-engineering of natural riboswitches include development of a 2,4-dinitrotoluene (DNT) responsive synthetic riboswitch in *E. coli* (Davidson et al., 2013) and PPDA (pyrimido[4,5-*d*]pyrimidine-2,4-diamine) binding mutant adenine aptamer with higher regulatory characteristics (Robinson et al., 2014). In addition, a recent study described a rational designed approach to re-engineer the PreQ1 riboswitch in *B. subtilis* into an orthogonal OFF-switch which effectively inhibited the transcription of desired genes (Wu et al., 2015). Klauser and colleagues reported a re-engineered hammerhead ribozymes which efficiently respond to aminoglycoside antibiotics (Klauser et al., 2015a). They first developed an *in vivo* selection protocol in *Saccharomyces cerevisiae* in order to examine large sequence spaces for optimized switches and used the developed system to increase the design possibilities for rendering the proposed sequence of ligand-dependent ribozyme (Klauser et al., 2015b). Development of new neomycin-dependent RNA modules with up to 25-fold gene expression switch was also achieved using this method. Beside to artificial aminoglycoside riboswitches, Jia and colleagues in 2013 declared the existence of aminoglycoside sensing riboswitch located in the upstream of aminoglycoside resistance genes including aminoglycoside acetyl transferase (*aac*) and aminoglycoside adenyl transferase (*aad*) (Jia et al., 2013). Consequently, a discussion raised by Breaker group that discovered regulatory elements were already known as key component of integrons functioning as recombination sites (*attI*) that facilitates the exchange of gene cassettes and possibly involved in antibiotic resistance (Roth and Breaker, 2013). Regardless of this discussion, some evidences are later provided in favor of the possibility of riboswitch-mediated regulation of bacterial resistance to aminoglycosides (He et al., 2013; Chen and Murchie, 2014).

Some methods are employed to improve the efficiency of engineered riboswitches. Emadpour and colleagues developed an RNA amplification-based system that extremely increases the efficiency of riboswitches and they applied the method in the chloroplasts of higher plants (Emadpour et al., 2015). In addition, some models can be developed to predict the regulation strength of engineered riboswitches such as the physics-based model introduced by Espah Borujeni and colleagues (Espah Borujeni et al., 2016). The model describes how riboswitch activation can be controlled by factors like aptamer structure, ligand affinity, switching free energy and macromolecular crowding. They validated the model experimentally using designed and characterized synthetic riboswitches containing aptamers bind to theophylline, fluoride, dopamine, thyroxine or 2,4-DNT and stimulated translation level up to 383-fold (Espah Borujeni et al., 2016). In another computational study, it was indicated that terminator hairpin stability and predicted folding traps have key roles on riboswitches transcriptional function using theoretically designed theophylline-dependent riboswitches (Wachsmuth et al., 2015).

Engineered riboswitches are usually designed to trigger a special response in the cell. In one of the studies, an entirely rational design strategy was developed to construct a novel artificial riboswitches working in a eukaryotic cell-free translation system (wheat germ extract). Accordingly, translation initiated by an internal ribosome entry site (IRES) due to the presence of a specific ligand (Ogawa, 2011). Furthermore, regulating virus genome can be done using riboswitches. Bell and colleagues demonstrated that gene expression from a positive-strand RNA virus genome can be regulated with riboswitches. Adding ribozyme–aptamers switches into the 3′UTR of the replicon led to a powerful control of expression through controllable cleavage of the genomic RNA as well as the subgenomic RNA (with both ON and OFF switches) (Bell et al., 2015). Strobel and colleagues introduced an artificial riboswitch (containing guanine-responsive aptazyme) to control transgene expression during adeno-associated virus (AAV) production in mammalian cells. The model was applied successfully in a mouse model of AAV-TGFβ1-induced pulmonary fibrosis (Strobel et al., 2015).

Artificial riboswitches have also potential to introduce unnatural phenotypes. In a study, a riboswitch was used to produce valuable metabolites. The *glmS* ribozyme was integrated into the 3′UTR of cytosine deaminase coding gene in *S. cerevisiae* to construct a suicide riboswitch. By adding fluorocytosine, the growth of the strain harboring the suicide riboswitch was stopped and metabolite level increasing made it recovered. By using this riboswitch, an efficient and high-throughput screening of different phenotypes can be possible (Lee and Oh, 2015).

5.3. Riboswitch-based biosensors

As a matter of fact, one of the main aims for developing engineered riboswitches is making new types of biosensors triggering by riboswitch-like functions. One of the recent approaches to make such systems was through making a Spinach-based riboswitch (You et al., 2015). Spinach is defined as an RNA aptamer that binds fluorophores similar to the hydroxybenzylidene imidazolinone (HBI) fluorophore in GFP (Song et al., 2014). You and colleagues invented a TPP Spinach riboswitch which allows the finding of agonists and antagonists of the TPP riboswitch using simple fluorescence signals. Based on this system, it is possible to monitor cellular metabolites in living cells (You et al., 2015).

Artificial riboswitches potentially may regulate genes with a desired molecule. Thus, they are anticipated to act as biosensors, in which the signal can be a measurable level like protein expression. Concerning all these attempts to construct artificial riboswitches, it is now achievable to use molecular recognition capability of riboswitches for development of biological sensors. Indeed, artificial riboswitches make it possible to control any gene expression with an arbitrary molecule. Expectedly, they could be several molecule dependent gene regulators *in vivo* (Suess and Weigand, 2008; Topp and Gallivan, 2010) as well as *in vitro* biosensors for detecting various molecules (Ogawa, 2011). Hence, an *in vitro* riboswitch biosensor should include a label related to conformation which emits a signal through ligand binding. In this kind of biosensors, an aptamer domain is derived from a natural or artificial riboswitch. Riboswitch biosensors can be applied in diverse situations and platforms such as solid supports (Breaker et al., 2008).

Using an aptamer domain of a TPP riboswitch by means of structure-switching and fluorescence signaling design, an *in vitro* biosensor for TPP was created (Lau et al., 2010). In addition, Fowler and colleagues constructed and applied novel series of riboswitch-based sensors to detect metabolites within bacterial cells (Fowler and Li, 2014). According to their hypothesis, it was assumed that a riboswitch could

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Table 1
Studies related to biosensor design based on riboswitches.

Ligand	Summary	Ref
Theophylline	A label-free and detector-free aptazyme-based riboswitch sensor was constructed for detecting the cofactor of the aptazyme.	Ogawa and Maeda (2007)
TPP	The dual genetic selection was carried out to design a bacterial riboswitch with improved sensitivity and the transformed *E. coli* with engineered riboswitches were characterized as whole-cell biosensors.	Muranaka et al. (2009)
B_{12}	An intracellular sensor of coenzyme B_{12} was described which make use of the molecular detection potential of a natural riboswitch.	Fowler et al. (2010)
Theophylline FMN Tetracycline Sulforhodamine B	Fully rational design strategy was conducted to construct novel artificial riboswitches that work in a eukaryotic cell-free translation system which are applicable as biosensors for detecting their ligands.	Ogawa (2011)
Cyclic dinucleotides	RNA-based fluorescent biosensors for cyclic di-GMP and cyclic AMP-GMP were designed by fusing the Spinach aptamer to different natural GEMM-I riboswitch. The biosensors showed fluorescence turn-on in response to cyclic dinucleotides in live cell imaging experiments.	Kellenberger et al. (2013)
Cyclic di-GMP	Minimalist approach toward *in vitro* quantification of c-di-GMP using the Vc2 riboswitch aptamer (GEMM-I class riboswitch aptamer upstream of a Tfox-like gene in *Vibrio cholerae*)	Kellenberger et al. (2015b)
Cyclic di-GMP	Design, make, and analysis of riboswitch–Spinach aptamer fusion RNAs for ligand-dependent activation of fluorescence *in vitro*.	Kellenberger and Hammond (2015)
Cyclic di-GMP	A label-free electrochemical biosensor design based on self-assembled riboswitch for sensitive and selective detection of c-di-GMP. The detection limit of is 50 nM	Xie et al. (2015)
Vitamin B_2	Making a whole-cell biocatalyst which detect the level of vitamin B_2 production from cellulosic biomass in *B. subtilis* using a designed FMN hammerhead riboswitch	Meyer et al. (2015)

be employed as a reliable, rather noninvasive intracellular metabolite sensor. In a study in 2010, such a sensor was constructed to detect adenosylcobalamin (AdoCbl) concentrations in the model Gram-negative bacterium *E. coli* (Fowler et al., 2010). The sensor was applied to conduct a detailed mechanistic study of a protein transport system within living cells (Fowler et al., 2013). In another study, a B_{12} riboswitch from *Propionibacterium freudenreichii* was cloned in GFP harboring vector in *E. coli* and used for determination of adenosylcobalamin level in fermented foods (Zhu et al., 2015).

Brief descriptions of some riboswitch-based biosensors are listed in Table 1.

5.4. Perspective

Riboswitches have been made a very applicable frame in order to design aptamers and biosensors. Before riboswitches, approaches such as Systematic Evolution of Ligands by Exponential Enrichment (SELEX) were developed to introduce desired ligand binding aptamers. However, Riboswitches introduced a natural template which is not only able to bind a specific compound but also trigger a detectable gene expression response. This unique characteristic attracts the attentions to this RNA element to be used as a template for engineered riboswitches which possess improved switching capability and/or new properties such as ribozyme-like activities. These genetic devices can be used as a powerful tool in desired biotechnological area and novel biosensors.

Despite the number of ways to design the desired switch, some points should be considered including the genes to be activated, strength of riboswitch binding sites and the strength of riboswitch regulation. Currently, it seems that most limitations in this field of riboswitches' applications have been overcome. As a result, switching resistance genes in pathogens such *Mycobacteria* (Seeliger et al., 2012) and even controlling virulence in viruses (Ketzer et al., 2014) are possible using riboswitch-based manipulations. Moreover, using these artificial systems to develop novel biosensors is a promising field of innovations as more explained in section 7.3.

6. Riboswitches as therapeutics targets

6.1. Background

According to FDA, antibiotic resistance is considered as one of the most serious public health issues all over the world which rises the need to develop novel antimicrobial drugs for resistant bacterial infections (Lewis, 1995). Hence, it would be useful to find new targets in cellular processes in order to identify novel receptors for antibiotics against resistant bacteria (Theuretzbacher and Toney, 2006). Riboswitches, as metabolite sensing domains of bacterial mRNAs, are promising drug targets for new types of antibiotics (Blount and Breaker, 2006; Winkler, 2005; Winkler and Breaker, 2003; Barrick and Breaker, 2007b).

Historically, at first this hypothesis was started from strikingly revealing of riboswitch-targeting mechanisms of four known antibacterial and antifungal agents. The compound roseoflavin binds directly to the aptamer domain of FMN riboswitch (Serganov et al., 2009) declining the expression of a reporter gene located downstream of an FMN riboswitch in *B. subtilis* (Ott et al., 2009; Lee et al., 2009). Pyrithiamine is pyrophosphorylated and then binds to TPP riboswitch (Sudarsan et al., 2005). L-Aminoethylcysteine (AEC) and DL-4-oxalysine are lysine derivatives which reduce the growth of some Gram-positive bacteria due to binding to lysine riboswitch (Blount et al., 2007). Mutation in all of mentioned riboswitches cause disruption in antimicrobial activity and inhibit their effects (Lee et al., 2009; Sudarsan et al., 2005; Patte et al., 1998).

Moreover, our group computational study showed a possible interaction between paromomycin and different riboswitches which relies on the structural similarity between riboswitches and ribosomal RNAs "16S A site". Docking calculations showed high affinity of paromomycin to different classes of riboswitches (Mehdizadeh Aghdam et al., 2014a). In another *in silico* study the affinity of other aminoglycosides such as gentamicin and kanamycin were elucidated through scoring function and molecular dynamic simulation. It was demonstrated that the binding energy and stability of riboswitches/aminoglycosides is comparable with riboswitches/natural ligands and 16S rRNA A site/aminoglycosides complexes (Mehdizadeh Aghdam et al., 2014b). This even likely unspecific binding was also considered in another study as a novel exceptional riboswitch/non-cognate ligand interaction using FRET assay method. Baird and Colleagues could show that out of expectations, kanamycin/c-di-GMP riboswitch interaction is specific and even modulating the conformation of the riboswitch in a way that inhibit the binding of cognate ligand (Baird et al., 2015). This interesting result in agreement with our computational studies is an initial step to investigate the possible interactions of aminoglycosides and other known RNA-binding antibiotics with riboswitches. It could also potentially be considered as possible alternative mechanisms of action as well as resistance of known

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antibiotics. In a very recent study, Dar and colleagues could discover new ribo-regulators in antibiotic resistance bacteria using developed conditional screening method, term-seq, explained in Section 4.4 (Dar et al., 2016). Term-seq mainly was applied on model bacteria *B. subtilis*, *L. monocytogenes*, *E. faecalis* and in the human oral microbiome. Identification of large number of regulators in model microorganisms and in the species of human microbiome indicates that ribo-regulation of antibiotic resistance genes is very common in Gram-positive bacteria. Additionally, new antibiotic-dependent regulators were discovered through this study. Mainly, a novel regulator in the 5′UTR of *lmo0919* gene, which encodes an ABC transporter, discovered in *L. monocytogenes* in response to lincomycin. Although, this discovery could not exactly identify the regulators as direct binders to the antibiotics, this study was another step to show the possibility of riboswitches' interaction with antibiotics.

There are some reasons of why riboswitches are attractive targets for new antibiotics. First of all, small molecule binding to a particular riboswitch is highly selective and specific (Blount and Breaker, 2006). Currently, most of manufactured antibiotics target ribosomal RNAs (rRNAs) (Sutcliffe, 2005). While, in contrast to rRNA domains, riboswitches are RNA receptors which specifically bind small molecules. Therefore, it is more possible to find a molecule that binds specific riboswitch rather than rRNA targets (Penchovsky and Stoilova, 2013). Secondly, apart from some exceptions, most of the riboswitches mainly exist in bacteria not in eukaryotes (Cheah et al., 2007). This distinction decreases cross-reactivity of bacterial riboswitch sensing ligands. Another reason is the associations of riboswitches with critical mRNAs which encode essential proteins for survival or/and virulence (Zhang and Zhang, 2008; Zhang et al., 2004). Consequently, at least for these reasons, targeting riboswitch could lead to cell death or debilitating.

6.2. Development of riboswitch-targeted antibiotics

Some methods have been employed for discovery of new drug compounds targeting riboswitches include structure-guided rational design, high-throughput screening methods and riboswitch specific assays. Using these methods, some antibacterial compounds with *in vivo* activity have been discovered. Table 2 shows some of the studies on the riboswitch based drug discovery.

6.2.1. Lysine riboswitch

In 2007 L-lysine analogs were identified which inhibit *B. subtilis* growth rate through binding to lysine riboswitch (Blount et al., 2007). Three out of 12 L-lysine analogs were evaluated to inhibit *B. subtilis* growth in minimal medium owing to riboswitch binding. At least partial mechanism of their function was determined as riboswitch-mediated growth repressors.

6.2.2. Guanine riboswitch

In a study on guanine riboswitch, sixteen analogs with modifications were tested for riboswitch binding, growth repression, and suppression of a reporter gene (Kim et al., 2009). Only three compounds completely inhibited growth rate and one of them was found to limit expression of a reporter gene under the control of guanine riboswitch. In another investigation on targeting the guanine riboswitch two pyrimidine analogs were predicted based on the crystal structure of riboswitch. *In vitro* demonstration was revealed that both pyrimidines inhibited *B. subtilis* growth in minimal media and changed reporter gene expression. One of these pyrimidine compounds (PC1) repressed the growth rate of *Staphylococcus aureus* (Mulhbacher et al., 2010). Hopefully, due to the sensitivity of multidrug resistant bacterial strains to the compound, it was presumed that its antimicrobial mechanism is not common with other known antibiotics. The evaluation of PC1 antibacterial effect on the bovine mastitis showed that it can reduce *S. aureus* concentrations. However, complete bacterial clearance was not achieved (Ster et al., 2013).

6.2.3. glmS riboswitch

glmS riboswitch has the unique capacity of mRNA cell-cleavage through binding to GlcN6P. A 5′-OH terminus RNA product of cleavage is recognized by RNase J1 which degrades *glmS* mRNA (Collins et al., 2007). The cognate ligand, GlcN6P, is the precursor of peptidoglycan biosynthesis making it an essential metabolite needed for bacterial cell-wall synthesis. In addition, most of the *glmS* riboswitches are present in Gram-positive organisms (Barrick et al., 2004). The Winkler group also verified that in the Gram-positive bacterium *B. subtilis*,

Table 2
Studies related to drug discovery based on riboswitches.

Riboswitch	Summary	Organism	Ref
Lysine	3 out of 12 lysine analog inhibit bacterial growth by riboswitch mediated repression	*Bacillus subtilis*	Blount et al. (2007)
Guanine	5 out of 16 guanine analogs reduce bacterial growth but just one of them repress guanine riboswitch regulated reporter gene	*Bacillus subtilis*	Kim et al. (2009)
Guanine	2 pyrimidine analogs predicted based on RNA structure repress *B. subtilis* growth, However just one (PC1) multi-drug resistant bacterial strains of *S. aureus*	*Bacillus subtilis* *Staphylococcus aureus*	Mulhbacher et al. (2010)
T-box	Screening derivatives of oxazolidinones led identification of one compound which reduce tRNA-dependent antitermination and another compound that increase tRNA-independent antitermination	*In vitro* assay	Anupam et al. (2008), Means et al. (2006)
glmS	High-throughput screening of ~1000 compounds utilizing FRET approach. Only glucosamine induced an efficient cleavage.	*In vitro* assay	Mayer and Famulok (2006), Blount et al. (2006)
TPP	A fragment-based screening using equilibrium dialysis identified 20 hits out of ~1300 fragments. However, may be due to small size of the fragments, they failed to affect gene expression.	*In vitro* assay	Cressina et al. (2011)
Guanine	Using PC1 (Mulhbacher et al., 2010) for therapy of *S. aureus* IMI in lactating cows. Treatment of infected cows with PC1 (0, 250, or 500 mg) lead to significant reduction in bacterial concentrations. However, only 15% of the PC1-treated cows underwent bacteriological cure.	*Staphylococcus aureus* (animal test)	Ster et al. (2013)
c-di-GMP	Binding characteristics of ligand analogs for representatives of c-di-GMP riboswitches were examined which support the possibility of drug design to target c-di-GMP riboswitches.	*Vibrio cholerae* *Clostridium difficile*	Furukawa et al. (2012)
glmS	Effect GlcN6P-analogs with phosphate mimicking groups at the 6-position on the activation of *glmS* riboswitch were studied.	*In vitro* assay	Fei et al. (2014)
FMN	The characterization of an analog of riboflavin, 5FDQD, and targeting FMN riboswitch was reported. 5FDQD has potent and rapid bactericidal activity against *Clostridium difficile* as well as less effect on culturable cecal flora	*Clostridium difficile*	Blount et al. (2015)
FMN	US pharmaceutical company Merck discovered “Ribocil” as a highly selective ligand and modulator of bacterial riboflavin riboswitches.	*Escherichia coli*	Howe et al. (2015)

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glmS riboswitch-ribozyme cleavage-inhibition leads to inability of sporulation or forming biofilms (Collins et al., 2007). Considering the importance of GlcN6P, inhibition of the *glmS* riboswitch could be lethal to microorganisms. Therefore, similar molecules to GlcN6P which trigger riboswitch activity have the potential to become antibiotics. In one of the first attempts, libraries of small molecules (Mayer and Famulok, 2006) and rationally-designed GlcN6P analogs (Blount et al., 2006) were screened using high-throughput assays for the identification of potential antibacterial compounds. However, the only compound identified was glucosamine which is very similar to the structure of GlcN6P. After identification of functional groups essential for GlcN6P recognition (Lim et al., 2006; Lunse et al., 2011; Wang et al., 2012) other GlcN6P analogs were examined. It was revealed that the phosphate of GlcN6P is important for binding to the ribozyme because of weaker activation by GlcN. Those analogs without the amine functionality do not cause *glmS* self-cleavage; however, those with raised amine pKa values lead to self-cleavage activity (McCarthy et al., 2005), and those with removed N lone pair through amide protection are inactive (Lim et al., 2006). Schwalbe and colleagues identified a GlcN6P analog named carba-GlcN6P (Schwalbe et al., 2007) with a two-fold decrease of activity compared to GlcN6P (Lunse et al., 2011). In another study, Fei and colleagues studied the GlcN6P analogs with phosphonate mimicking groups at the 6-position. This approach could reduce the phosphatase effect and two analogs (malonyl ether and the sterically true phosphonate) showed significant activity, however with 1/7 activity of natural ligand (Fei et al., 2014).

6.2.4. FMN riboswitch

In 2015, two important studies were published which introduced two antibacterial compounds based on FMN riboswitch. Blount and colleagues reported the characterization of an analog of riboflavin, 5FDQD, targeting FMN riboswitch. 5FDQD has potent and rapid bactericidal activity against *Clostridium difficile*. However, it causes less change of culturable cecal flora leading to low possibility of infection recurrence (Blount et al., 2015). In another interesting study, US pharmaceutical company Merck discovered "Ribocil" as a highly selective ligand and modulator of bacterial riboflavin riboswitches. Ribocil was found using phenotypic screen assay and showed a structurally different synthetic mimic of FMN to inhibit riboswitch-mediated *ribB* gene expression and consequent bacterial cell growth. This compound was found after screening a library of about 57,000 synthetic small molecules with antibacterial activity on *E. coli*. "Ribocil" showed complete inhibition of bacterial cell growth especially in the presence of exogenous riboflavin addition. In addition, Ribocil showed higher selectivity in its riboswitch inhibiting effect compared to Roseoflavin or other similar ligands. This was the first riboswitch-binding compound that is not a structural analog of a natural ligand, and thus, Ribocil did not repress other pathways involving riboflavin and FMN metabolism leading to less toxic effects in mice (Howe et al., 2015). This discovery can be a turning point in riboswitch-based antibiotic discovery since a phenotypic screen led to the introduction of a synthetic mimic of FMN which can act beyond an antimetabolites antibacterial activity against the most dangerous type of drug-resistant pathogens. Moreover, it was a promising report to show that riboswitches are "druggable" targets in synthetic chemical biology.

6.3. Screening of compounds

Riboswitches can be simply checked out for specific ligand binding in various *in vitro*, *in vivo*, and *in silico* high-throughput screening (HTS) methods (reviewed in Penchovsky and Stoilova, 2013). Followings are some examples using HTS for different types of riboswitches.

6.3.1. glmS riboswitch

In a study based on high-throughput discovery of activators of the *glmS* riboswitch, ligand-induced self-cleavage resulted in a short fluorescent oligonucleotide which cause augmented polarization-based decorrelation (Mayer and Famulok, 2006). In another study using FRET (fluorescence resonance energy transfer), approximately 1000 compounds were screened for activating *glmS* riboswitch. Nevertheless, only glucosamine could cause efficient cleavage (Blount et al., 2006). Carba-sugars as leading compounds for antibacterial drugs were also discovered by means of HTS on *glmS* riboswitch (Lunse et al., 2011).

6.3.2. c-di-GMP riboswitch

Furukawa and colleagues developed an assay for rapid screening of c-di-GMP analogs. The assay device for *in vitro* selection consisted of a c-di-GMP-I riboswitch aptamer and a hammerhead ribozyme (Furukawa et al., 2012).

6.3.3. SAM-I riboswitch

In another study, Hickey and colleagues developed high throughput screening for SAM-I riboswitch binders by synthesizing a fluorescent analog of *S*-adenosyl methionine and using a fluorescence polarization assay (Hickey and Hammond, 2014).

6.3.4. T-box riboswitch

Liu and colleagues designed an effective cascade of screening assays which linked the changes of ligand binding to the fluorescence of an attached dye or the fluorescence anisotropy of an RNA complex. Fluorescence-monitored screening assays were employed to identify T-box riboswitch antiterminator element targeting compounds (Liu et al., 2015).

6.3.5. preQ1-I riboswitch

In another approach to describe a screening method, an assay which monitor the riboswitch induced _1 ribosomal frameshifting (_1 FS) efficiency was developed. The model target was preQ1-I riboswitches in a mammalian cell-free lysate system. Some advantages were declared for this method such as the probability of HTS by fusing to suitable reporters and on-line monitoring of possible side effects on the eukaryotic translation machinery by using a rabbit reticulocyte cell-free lysate (Yu and Olsthoorn, 2015).

6.3.6. TPP riboswitch

Another capable strategy for drug discovery based on riboswitches is fragment-based screening (Chen et al., 2010). Applying this method against *E. coli* TPP riboswitch, 20 hits out of approximately 1300 fragments were identified (Cressina et al., 2011). Afterwards, the study on the four hits showed that the recognizing part of the pyrophosphate of TPP rearranges into a different structure from the complex of the TPP riboswitch/thiamine (Warner et al., 2014). Therefore, the study approved the possibility of fragment-based drug discovery for riboswitches.

6.3.7. In silico HTS

Lafontaine and colleagues employed molecular docking to screen a virtual library and find PC1 binding to the guanine riboswitch (Colizzi et al., 2014). In order to do a computational HTS, primarily validated 3D structure of desired riboswitch (bound and unbound) should be provided (Subramaniam et al., 2008). Subsequently, a large virtual chemical library of different structures similar to the preferred riboswitch is built. Then, using suitable docking software, such as DOCK, selected virtual chemicals can be checked out for binding to the ligand binding pocket of riboswitches. Acquired compounds through this method could be more examined by *in vitro* as well as *in vivo* techniques. Finally, selected compounds will become a lead compound applied in preclinical and clinical trials in order to be approved as effective medicines.

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Table 3
Some important patents regarding riboswitches.

Classification	Title	Inventors	Ref.
Patents of novel riboswitches, structures, methods, cognate and non-cognate ligands	Riboswitches, methods for their use, and compositions for use with riboswitches	Breaker RR et al.	Breaker et al. (2004)
	Riboswitches, structure-based compound design with riboswitches, and methods and compositions for use of and with riboswitches	Breaker RR et al.	Breaker et al. (2008)
	glmS riboswitches, structure-based compound design with *glmS* riboswitches, and methods and compositions for use of and with *glmS* riboswitches	Breaker RR et al.	Breaker et al. (2010)
	Method for identifying SMK box riboswitch modulating compounds	Ke A et al.	Ke (2010)
	Riboswitches	Ban N et al.	Ban and Thore (2011)
	Lysine riboswitch and compositions and uses thereof	Batey RT et al.	Breaker et al. (2013a)
	SAM-II riboswitch and uses thereof	Batey RT et al.	Batey and Gilbert (2011)
	S-adenosyl-(L)-homocysteine (SAH) riboswitches and compositions and uses thereof	Batey RT et al.	Batey et al. (2014)
	Crystalline xpt guanine riboswitch from Bacillus subtilis and structure-based compound identification employing said riboswitch	Breaker RR et al.	Breaker et al. (2013d)
	Methods and reagents for analyzing riboswitches using FRET	Blanchard SC et al.	Blanchard et al. (2012)
	GEMM riboswitches, structure-based compound design with GEMM riboswitches, and methods and compositions for use of and with GEMM riboswitches	Strobel SA et al.	Strobel et al. (2012)
	Cyclic di-GMP-II riboswitches, motifs, and compounds, and methods for their use	Breaker RR et al.	Breaker et al. (2013b)
	Glycine riboswitches, methods for their use, and compositions for use with glycine riboswitches	Breaker RR et al.	Breaker et al. (2013a)
	PreQ1 riboswitches and methods and compositions for use of and with preQ1 riboswitches	Breaker RR et al.	Breaker et al. (2013c)
	Modulators of RNA riboswitches	Patel D et al.	Patel et al. (2011)
Patents of engineered riboswitches	Riboswitch based inducible gene expression platform	Bertozzi CR et al.	Bertozzi et al. (2012)
	Two-way, portable riboswitch mediated gene expression control device	Huang JD et al.	Huang and Jin (2013)
	EMP2: Ethyl-Methyl, di Methyl, tri Methyl Pyruvate Acid Esters: A Tool for Regulating HbA1c and a Riboswitch Activator	Fitzgerald JJ et al.	Fitzgerald et al. (2013)
	Thiamine pyrophosphate (TPP) riboswitch mutants producing vitamin B_1 enriched food and feed crops	Aharoni AS et al.	Aharoni et al. (2013)
	Method for screening for high L-tryptophan producing microorganisms using riboswitch	Jung GY et al.	Jung et al. (2013)
Patents of riboswitch-based drug design	T-box riboswitch-binding anti-bacterial compounds	Agris PF et al.	Agris (2015)
	Guanine riboswitch binding compounds and their use as antibiotics	Mulhbacher J et al.	Mulhbacher et al. (2015)

6.4. Limitations and perspective

In the first look, RNAs do not provide a suitable frame for drug targeting because of limited monomers and negative charges which make the specific targeting a challenging job. However, some antibiotics like aminoglycosides or macrolides target rRNAs. Also, RNA aptamers and riboswitches were shown to be a specific target of metabolites. Therefore, riboswitches could be promising targets for drug design, though there are still some limitations and issues in this regard.

One approach could be to start from natural ligands and change the moieties to achieve an active synthetic compound. However, analogs derived from this way have the risk of interfering with the enzymes of metabolic pathways. At this point, some scientists suggest the dissimilarity between artificial compound and natural compounds. However, pure dissimilarity expectedly causes unacceptable produced compounds. Nonetheless, one promising method to develop such compounds is fragment-based screening as it screens the libraries with smaller size of natural ligand like what has been done on TPP riboswitch (Cressina et al., 2011). Also, a recent phenotypic screen which yielded a synthetic compound targeting FMN riboswitch, named Ribocil, demonstrated that a suitable screening has a key role in order to find an appropriate lead compound to design antibiotics.

Beside to the limitations around screening and finding an active compound, another important issue is not fully understanding the regulation mechanisms around riboswitch binding metabolites. For instance, some antisense small RNA-based regulation of *glmS* gene could be one of the reasons why achieving a suitable antibiotic targeting *glmS* ribozyme/riboswitch has not been completely successful. On the other hand, some possible riboswitch-mediated resistance mechanisms, as known for aminoglycoside resistance (Chen and Murchie, 2014), can be another limiting factor. Attention should be paid to this fact that bacteria could develop riboswitches against antibiotics and this can stop a therapy supposed to halt a riboswitch.

7. Riboswitch-related patents

Since about a decade ago, when for the first time it was approved that some mRNAs carry riboswitches (Nahvi et al., 2002; Mironov et al., 2002; Winkler et al., 2002a; Winkler et al., 2002b), more than 30 riboswitch classes have been experimentally confirmed. Regarding emerging applications of riboswitches, several patents have been filed to cover related innovations for the past years. As discussed before, specific non-coding RNA elements have been used as models for artificial switches and synthetic gene controlling devices. Moreover, riboswitches' unique structure in selective binding to vital metabolites in the cells made them an attractive target to design novel antibiotics. These interesting potentials of riboswitches led the scientists to make various innovations and consequently, numbers of novel applied studies in this area have been patented. In order to find the related patents, United States Patent Office patent search page (http://www.uspto.gov/patft/index.html) and Free Patents Online database (http://www.freepatentsonline.com/) were used and key word “riboswitch” was searched usually in the specification part.

Some of the most important patents which cover different fields of riboswitches are listed in Table 3. Briefly, first type of patents in this realm of innovations are often related to different methods of riboswitches discovery, constructs and organisms harbor them naturally or artificially as well as some suggested unnatural active compounds on riboswitches. These types of patents are mostly invented by Breaker group (Breaker et al., 2008; Breaker et al., 2009; Breaker et al., 2004). Although these types of patents have still been continued to recent years, nowadays most patents are focused on applications of riboswitches such as designing genetic devices and introducing compounds as

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riboswitches' inhibitors/activators with antibacterial function. As a result, here we can generally divide patents in three below groups.

7.1. Patents of novel riboswitches, structures, methods, cognate and non-cognate ligands

Most of these types are patented by Breaker group at Yale University. To give a few examples, glycine, lysine, c-di-GMP, c-di-GMP-II, Fluoride, *glmS*, preQ1 riboswitches are described in patent applications numbers US20130029342 A1 (Breaker et al., 2013a), US20110206693 A1 (Batey and Garst, 2011), US20120107331 A1 (Strobel et al., 2012), US20130143955 A1 (Breaker et al., 2013b), US20150023889 A1 (Breaker et al., 2015), US20100324123 A1 (Breaker et al., 2010), US20130012527 A1 (Breaker et al., 2013c), respectively. In these patents, representatives, structure, mechanisms and methods involved with natural riboswitches are presented. In addition, fused constructs containing desired riboswitch and a reporter gene were provided in order to evaluate cognate and non-cognate ligands. As an example, in patent application number US20100221821 A1, TPP riboswitch located in eukaryotes with the mechanism of alternative splicing was presented. Moreover, some other riboswitch classes and riboswitch-like elements (called orphan riboswitches) were patented in patent application number 20120321647 A1.

Additionally, there are some patents regarding method development in this area. For instance, there is a method development for identifying a compound that alters gene expression under the control of SMK box riboswitch. Accordingly, structural atomic positions of free state SMK box riboswitch as well as bound state structure were included (Ke, 2010). A recent method development was patented in which different types of isolated riboswitches join to FRET pair of fluorophores capable of differentiating changes in regulatory interactions. These riboswitches also contain an immobilization moiety to enable surface immobilization or immobilization to a solid support. One application of this patent is to monitor the conformational changes and/or the mechanism of regulation of riboswitches. Another application could be the usage of this method to identify new compounds interfering with the riboswitch by smFRET imaging (Blanchard et al., 2012).

7.2. Patents of riboswitch-based drug design

7.2.1. glmS riboswitch/ribozyme

Breaker group in patent application number US20100324123 A1, patented various derivatives of GlcN6P as the cognate ligand of *glmS* riboswitch/ribozyme and potential lead compound for antibiotic design. Accordingly, cyclohexane compounds as antibacterial candidates were examined with the idea of affecting on *glms* riboswitch/ribozyme and Carba-GlcN6P with antibacterial activity was patented with patent application number of US20140066409 A1 by Mayer and colleagues.

7.2.2. Fluoride riboswitch

According to the fact that there is a riboswitch-based fluoride regulation in the cell and fluoride is a toxic compound for the cells, Breaker and Li patented some antibacterial and antifungal agents such as polyene (as permeabilizing agents) to enhance fluoride concentration in the cell (US20150030701 A1). Therefore, a fluoride riboswitch-beta galactosidase construct was used in order to examine the fluoride concentration increase by the effect of the compounds on the expression of the reporter gene.

7.2.3. Guanine riboswitch

Patent application number US20150150890 A1 considered novel antimicrobial compounds derivatives of non-ribosylable ligand of guanine riboswitch with the mechanism of *guaA* gene inhibition through binding to a guanine riboswitch. These compounds were used for preventing or treating the infection caused by a pathogen harboring guanine riboswitch-mediated *guaA* gene regulation. Also, disinfection, sterilization and antisepsis of the same type of bacteria as well as treating MDR infections were evaluated by invented compounds (Mulhbacher et al., 2015). As an important point, it was shown that *S. aureus* did not develop resistance toward compound 2,5,6-triaminopyrimidine-4-one (known as PC1).

7.2.4. T-box riboswitch

In patent application number WO2015013431 A1, inventors developed compounds target tRNA-dependent riboswitches of *aaRS* gene mostly found in Gram-positive bacteria. Compounds were screened computationally first and then tested to measure MIC and MBC on *S. aureus* and *B. subtilis* (Agris, 2015). Compounds PKZ6 and PKZ18 had the most effective antibacterial activity.

7.3. Patents of engineered riboswitches

Chimeric riboswitches including an aptamer domain from one source and expression platform from another source are interesting switching devices to design biosensors. Using such an approach, in patent number US9222093-A, so called two-way engineered riboswitch was designed which is in ON state by default and changed to OFF state with theophylline and go back to ON state with IPTG (Huang and Jin, 2013). An engineered riboswitch can also be used to modify metabolism of an organism. Following this idea, TPP riboswitch of *thic* gene of *Arabidopsis thaliana* was engineered in order to be blocked and thiamine level of the plant increased. These modified plants can be used as enriched food supply (Aharoni et al., 2013).

In patent number USRE43000 E1 (Gaur and Pillai, 2011), an engineered riboswitch was constructed to regulate pre-mRNA splicing. High affinity theophylline binding aptamer was inserted into the pre-mRNA. In the presence of theophylline, RNA splicing can be modulated. Based on this invention, there are other innovations in this patent such as theophylline-dependent riboswitches which alter RNA splicing, identifying theophylline-dependent riboswitches and procedures of treating abnormal RNA splicing caused diseases.

8. Conclusion

More than a decade after first riboswitch discovery, we know more about these RNA regulators. More riboswitch candidates have been assigned with definite molecules just recently and new gates are open now to find out more about these special elements in live world. Structural-based analysis, both computationally and experimentally, have been carried out to understand the mechanism of riboswitches more efficiently. In addition several applications are developed using natural or artificial riboswitches.

Considering specificity and selectivity of riboswitches to bind different kinds of metabolites, they are attractive tools to be applied as living biosensors. Besides, artificial riboswitches can be employed to regulate the expression of target genes through binding the molecules (Topp and Gallivan, 2010). Pioneer efforts in this area make it easier to develop new probes employed to detect important molecules. Riboswitch-based sensors are simple, flexible and reliable enough to exploit a large list of possible applications for them. Therefore, there is a bright future for them as precious tools in biological studies.

In the other side, riboswitches are very special drug targets since they have been considered as small molecule sensors in the cell (Blount and Breaker, 2006). They seem to be very capable targets for the discovery and development of new antibiotics against resistant bacteria strains. A problem in riboswitch sensing drug discovery is to find out whether *in vitro* effective compounds could exert their effects *in vivo*. Another issue is cross-reactivity and toxicity for potential riboswitch targeting drugs. Compounds such as pyrithiamine (Koedam, 1958) and AEC (Di Girolamo et al., 1986) which target riboswitches have been revealed to interact with mammalian proteins. However, different recognition mechanisms of riboswitches and

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proteins as well as absence of known riboswitches in human reduce the probability of cross-reactivity of new riboswitch-targeting compounds. Antibiotic resistance could be another concern because of riboswitch mutations. However, this resistance may be less occurred in riboswitches rather than target proteins. Recent publication of US pharmaceutical Merck Inc. reporting "Ribocil" (Howe et al., 2015) as a FMN riboswitch targeted antibiotic with strong bactericidal effect and less side effects shed light to the pathway of riboswitch-based antibiotic discovery and it is hopefully achievable to develop new types of antibiotic compounds against resistant infections due to the presence of multiple riboswitch classes in different pathogens.

Selective targeting of riboswitches with synthetic compounds is still is in its initial stages. After about 15 years of riboswitches introduction to molecular biology world, very few compounds are known to be active as antibacterial compounds *in vivo*. Moreover, the interaction possibility between known antibiotics such as aminoglycosides and riboswitches make them a complicated platform for drug design. Enlightening studies on the mechanisms and structures of riboswitches as well as investigation on their probable involvement in antibiotic resistance can accelerate the process of riboswitch-based antibiotic design.

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