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ORIGINAL ARTICLE

RNA's coming of age as a drug target

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Abstract After being perceived for a long time merely as an intermediate between DNA (the depository of the genetic information) and proteins (the macromolecules that work inside a cell), RNA is now the center of attention in biomedical research. RNA's boost in fame is in part attributable to the discovery of its role in controlling the expression of certain genes through direct sensing of small molecules. In addition, the RNA component of the protein synthesis machinery is the prime target for most antibiotics. Molecular recognition principles that underlie RNA–ligand interactions in these systems emerged from relentlessly refined structural, thermodynamic and kinetic studies. Together, they keep informing the design of novel drugs that are much needed to help alleviate the current resistance crisis.

Keywords Aminoglycoside · Antibiotic · Recognition · Ribosome · Riboswitch · RNA

The omnipresence of non-coding RNAs

Considering that over 50% of the genomic DNA of complex organisms is transcribed into RNA, whereas under 2% of this DNA actually encodes proteins [1], it is not too unexpected that RNA is found at many key regulatory steps of a cell's life [2]. The fundamental process of genetic expression, which mainly comprises (1) DNA replication, (2) transcription of DNA into a precursor messenger RNA (pre-mRNA), (3) splicing of this pre-mRNA to a mature mRNA, and (4) translation of the mRNA sequence into a protein sequence, is highly controlled by a variety of non-coding RNAs (ncRNAs). For example, long ribosomal RNAs (rRNAs, from 120 to 2,300 nucleotides) assemble with over 55 proteins to constitute the ribosomal particles that catalyze protein synthesis (for two recent reviews on ribosome assembly and function, refer to [3, 4]). On the other hand, short RNAs (about 21–28 nucleotides) such as short interfering RNAs (siRNAs) and micro RNAs (miRNAs) can base pair with a specific mRNA to repress its translation or guide its degradation (see reviews in [5, 6]). These ncRNAs are only two examples in this universe of RNA-mediated regulation where the number of RNAs being discovered and characterized is rapidly increasing [7, 8].

To carry out these multiple functions, RNAs typically adopt precise three-dimensional architectures, much like proteins do. The first illustration of that aspect was the now characteristic L-shaped structure of transfer RNA (tRNA) solved in the 1970s–1980s [9–11] (Fig. 1), which offered the first structural clues into the role of tRNAs as molecular adaptors during protein synthesis. Their structure enables tRNAs to first get aminoacylated by a protein enzyme, and then to recognize a particular triplet of nucleotides on the mRNA at the decoding site, while matching the corresponding amino acid into the protein synthesis site of the ribosome [12]. Ensuing findings showed for example how some RNA molecules, belonging to the family of group I introns, can accommodate ligands and metal ions in structured binding sites (Fig. 1). Group I introns are metalloenzymes made of RNA—also called *ribozymes*—that require

Dedicated to Prof. Jack Harrowfield and Dr. Jacques Vicens—my dad!—on the celebration of their 65th birthday

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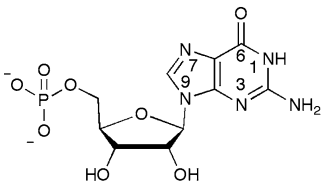
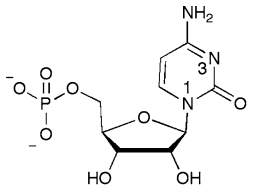
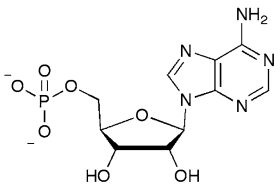


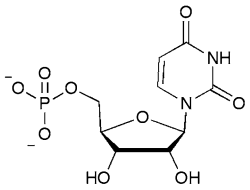
1, guanosine-5'-monophosphate (GMP)



2, cytidine-5'-monophosphate (CMP)



3, adenosine-5'-monophosphate (AMP)



4, uridine-5'-monophosphate (UMP)

binding of a guanosine cofactor (or one of its phosphorylated derivatives like **1**) and of magnesium ions in order to catalyze transesterification reactions involving phosphodiester bonds [13–17]. These examples illustrate how RNA building blocks **1**–**4**, in spite of lacking the chemical diversity of amino acids (e.g., guanidinium group in arginine, carboxylic group in aspartic acid, thiol group in cysteine), can assemble in three dimensions to form complex architectures that bind small effectors or substrates in order to assist or catalyze chemical reactions (Fig. 1).

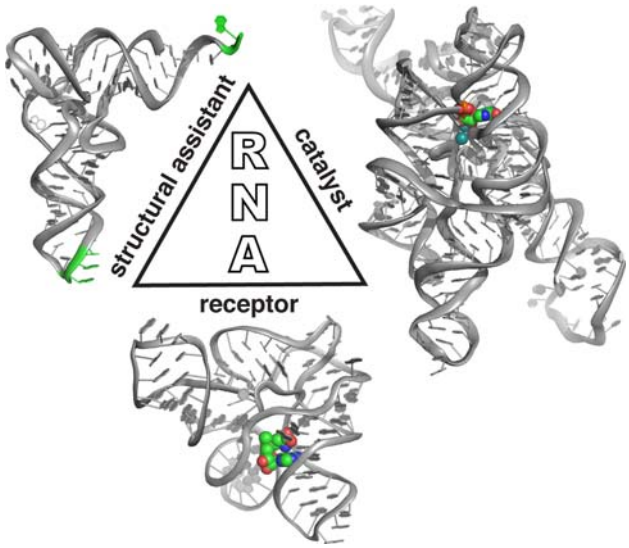


Fig. 1 RNA in three dimensions. Phenylalanine tRNA (76 nucleotides; PDB ID 1EVV; *left*), a group I intron (206 nucleotides; PDB ID 1ZZN; *right*), and a riboswitch (94 nucleotides; PDB ID 2GIS; *bottom*) are drawn to scale with each other using a ribbon representation in PyMOL (Warren L. DeLano "The PyMOL Molecular Graphics System", DeLano Scientific LLC, San Carlos, CA, USA, http://www.pymol.org). The following parts are highlighted in *green*: (i) the three nucleotides in the anticodon loop of tRNA interacting with the mRNA (not shown), as well as the nucleotide at the 3′ end which serves as the site of attachment to an amino acid; (ii) the terminal guanosine of the group I intron bound to the G-binding site (shown as spheres); and (iii) the ligand bound to the riboswitch (shown as spheres). The two magnesium ions involved in catalysis of the group I intron are shown as teal spheres. In this and subsequent figures, PDB ID codes refer to the structure coordinates retrieved from the RCSB PDB Protein Data Bank at http://www.rcsb.org/pdb/

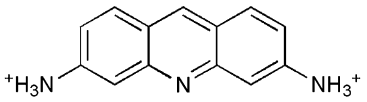
Non-coding RNAs as unsuspected but now much sought-after drug targets

Most antibiotics—like aminoglycosides—target bacterial ribosomal RNA

RNA has been known to bind chemotherapeutic agents like the intercalating agent proflavine **5** since the late 1960s [18, 19]. Two decades later, the finding that most antibiotics in clinical use target bacterial ribosomal RNA came as a breakthrough [20–22]. Subsequently, aminoglycoside antibiotics—such as paromomycin **6**, neomycin **7** and tobramycin **8**—gained a significant attention from their ability to bind to small fragments of rRNA (27–49 nucleotides in size) in the same manner as they would to the complete ribosome [23–25]. This finding enabled seminal NMR studies of antibiotic-RNA complexes [26, 27].

More recently, another turning point appeared with the publication of structures of various ribosomal subunits in complex with different classes of antibiotics, including paromomycin **6** but also for example erythromycin **9** and pactamycin **10**. These structures provided much-awaited insights that brought our understanding of the mechanisms of antibiotic action to the atomic level [28–33]. Specifically, aminoglycoside antibiotics were revealed to stabilize the conformation of two adjacent adenosines at the decoding center that normally occurs only in presence of a cognate recognition between the mRNA codon and the tRNA anticodon [28, 34, 35]. These results offered molecular details of the long-known misreading provoked by this family of antibiotics [36, 37].

Additionally, this ensemble of structural studies of ribosomal particles bound to small molecules expanded our knowledge of the molecular recognition principles that permit RNA binding to the point that structure-based drug design strategies aimed to target RNA became realistic [38]. To further aid such ambition, parallel efforts contributed to delineate principles for molecular recognition of **6**–**8** and related antibiotics by the ribosomal decoding site. For example, crystal structures were solved of aminoglycoside antibiotics bound to ribosomal RNA fragments [39–42] or to complete ribosomes [43, 44]. Importantly, the energetics and the dynamics associated with antibiotic binding emerged from thermodynamic analyses [45–48] and molecular dynamics simulations [49, 50]. As a result,



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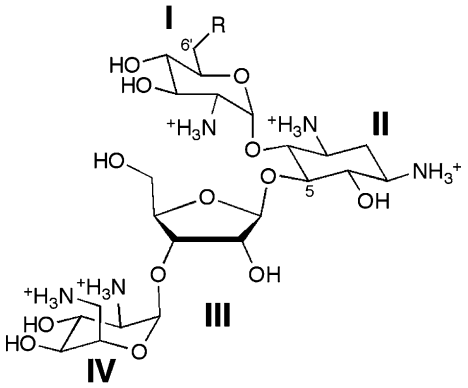
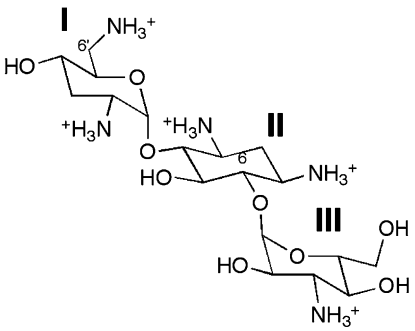


6: R = OH
7: R = NH_3^+



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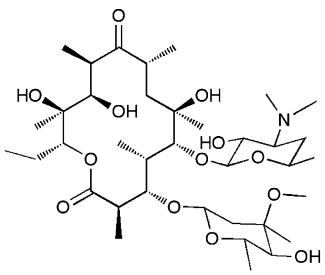

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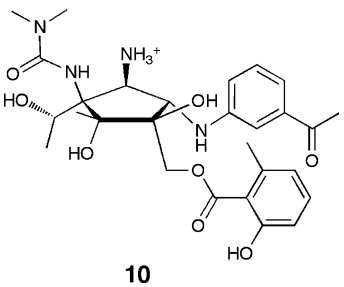

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the bacterial ribosome—and particularly ribosomal RNA and the decoding site—went back to the front seat of ideal targets for novel antibiotics [38, 51, 52]. However, the pressure is now strong to expand the pool of antibiotics in a timely manner, so that more weapons would be at our disposal to fight the escalating proportion of infections caused by resistant bacteria [53]. To multiply the chances of breakthrough, the search for new antibiotics is coupled to the search for new targets. A recently identified family of ncRNAs represents another promising gateway into unique RNA targets and antibiotics.

Riboswitches: an original entry into RNA targeting

Although the proportion of DNA encoding proteins is much higher in bacterial than in eukaryotic genomes [54], regulatory ncRNAs also exist in bacteria [7, 55]. In particular, distinctively structured mRNA regions termed "riboswitches" serve as receptors for a variety of small metabolites (nucleotide and vitamin derivatives) found inside many bacteria [56, 57] (see an example in Fig. 1). The binding of such a metabolite to a particular riboswitch—generally located upstream of a protein coding region—induces a type of allosteric effect that is associated either with sequestering or with releasing a stretch of nucleotides that controls the expression of the downstream gene. Over 20 families of riboswitches in the three domains of life (Bacteria, Archaea, Eukaryotes) have now been reported to be responsible for the control of diverse cellular processes, principally transcription and translation [58]. Furthermore, the percentage of genes controlled by riboswitches in a single genome has been estimated to be over 4% in certain bacteria [59], indicating that direct metabolite-sensing by RNA constitutes a widespread alternative to the classical protein-mediated control of genetic expression [60, 61].

Remarkably, the structures of almost all the exemplars of each riboswitch family (see review in [58]) have been solved in the seven-year time span since the identification of the first two riboswitches [56, 57]. The implications of this formidable concerted achievement are two-fold. First, RNA drug designers now have at their disposal an original treasure chest of highly structured RNA molecules that selectively recognize specific ligands. Second, riboswitches represent a new gateway into RNA targeting, since they evolved precisely to serve as receptors of small molecules, in contrast to ribosomal RNA [62]. Together, these observations suggest that riboswitches have a strong potential to enable a quantum leap in the development of novel antibiotics that target RNA [62, 63].

Because riboswitches make for a tremendous chance to apply what has been learned from investigating other RNA–ligand complexes, the offer to write this review came

as a timely opportunity to assess the current comprehension of molecular recognition of small molecules by ncRNAs. Therefore, in the sections that follow, I compare ligand recognition by natural RNA receptors in rRNA–aminoglycoside complexes and in riboswitch–effector complexes. The emphasis here is on the inter-molecular interactions that facilitate a selective binding, as well as the energetics, the dynamics and the kinetics associated to the binding event, in accord with the definition of *molecular recognition* suggested by J.-M. Lehn: "*Molecular recognition* is defined by the *energy* and the *information* involved in the *binding* and *selection* of substrate(s) by a given receptor molecule; it may also involve a specific *function*" [64, 65]. Furthermore, I illustrate how the knowledge of molecular recognition by RNA has informed the design of novel ligands. Finally, I highlight some of the most promising paths that could lead to future breakthroughs in the discovery of drugs that target ncRNAs.

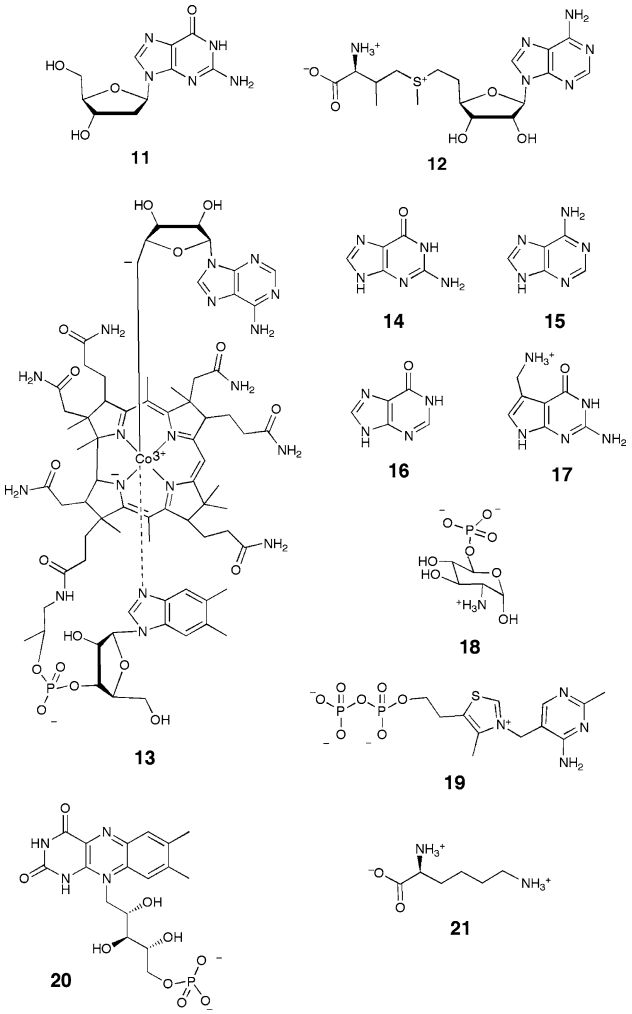
How does molecular recognition happen with RNA?

Ligands mimic RNA building blocks

RNA building blocks **1**–**4**, or nucleotides, are composed of three parts: (i) a nitrogenous base, (ii) a ribose sugar, and (iii) a phosphate group that is negatively charged at physiological pH (~7.4). Unlike protein-binding ligands, whose structures do not necessarily resemble that of amino acids, natural RNA-binding ligands often consist of nucleotides and their derivatives (e.g., 2′-deoxyguanosine (dG, **11**), *S*-adenosyl-methionine (SAM, **12**), coenzyme B12 (deoxyadenosylcobalamin, **13**). The limited range of RNA functional groups could explain this particular feature, as the non-covalent interactions conducive to binding should be facilitated between partners having similar geometries and interaction sites. Furthermore, most of the remaining compounds have at least one part in common with a nucleotide. For example, guanine (G, **14**), adenine (A, **15**), hypoxanthine **16** and prequeosine (preQ1, 7-(Aminomethyl)-7-deazaguanine, **17**) are isolated purine bases, **6**–**8** and related aminoglycosides possess a ribose sugar modified by several 6-membered rings, while glucosamine-6-phosphate ($GlcN_6P$, **18**), thiamine pyrophosphate (TPP, vitamin B1 monophosphate, **19**), and flavin mononucleotide (FMN, vitamin B2 phosphate, **20**) all contain phosphate groups. Finally, some of these compounds have also developed alternative chemical strategies to favor interactions with RNA, for example: (1) Lysine (Lys, **21**) and **12** contain carboxylic groups that could substitute for phosphate groups; (2) **5**, **10**, **19** and **20** contain planar rings that resemble the nitrogenous bases; (3) **6**–**8**, **10**, **12**, and **18**, among others, possess at least one positive charge to help binding to negatively charged RNA. As a consequence of these chemical properties, this somewhat mixed collection of RNA-friendly compounds interact with RNA using a combination of various inter-molecular forces, reminiscent of what had been observed earlier in tertiary interactions that stabilize RNA structures [9–11, 66] and between adenine derivatives and synthetic receptors [67].

Direct hydrogen bonds are essential for specificity and selectivity

Hydrogen bonding is the principal means toward both geometrical recognition and proper orientation of the ligand in the binding pocket of its receptor. For example, out of nine donor and acceptor positions of hydrogen bonds in guanine, eight are involved in the formation of direct hydrogen bonds with nucleotides from the binding pocket of the purine riboswitch [68, 69]. Three of these occur between the Watson–Crick faces of the guanine ligand and



the cytosine at position 74, exactly as in a typical Watson–Crick G=C pair (Fig. 2a). Remarkably, replacing C74 by a uracil changes the specificity of that riboswitch to adenine through the formation of a Watson–Crick A–U pair [70, 71] (Fig. 2b). Removal of a single hydrogen bond at the interface between the ligand and the pyrimidine at position 74 decreases the free energy of binding by 1.5–2.0 kcal $\cdot$ mol^{-1} (depending on the bond being disrupted) [71]. In fact, hydrogen bonds involving the 6-membered ring of the purine are more important for ligand binding than that involving the 5-membered ring [71, 72]. Furthermore, removal of all but two hydrogen bond donors and acceptors as in benzimidazole **22** induces a complete loss of binding. Together, these studies illustrate that while hydrogen bonds are critical for ligand recognition, not all hydrogen bonds contribute equally to the binding affinity.

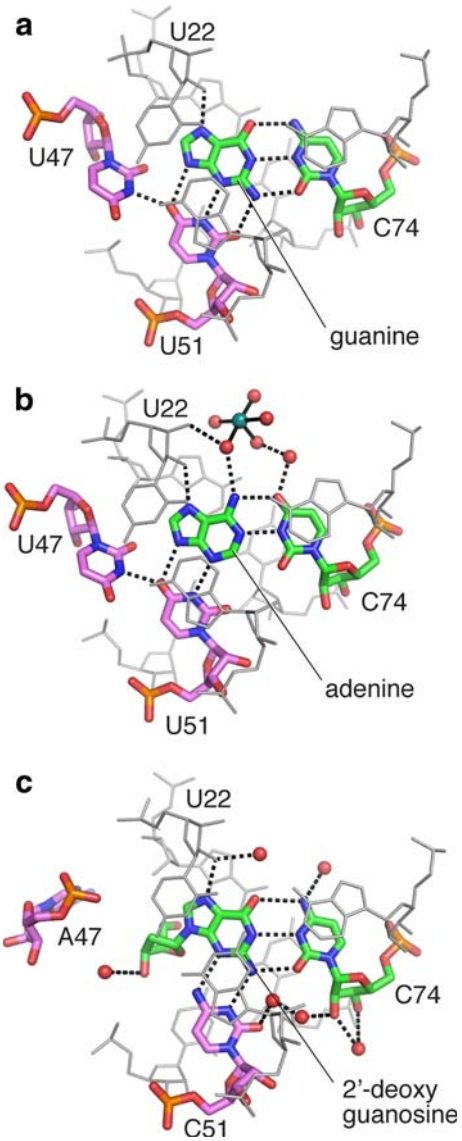
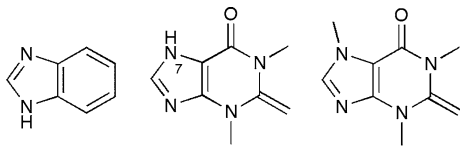


Fig. 2 Specificity determinants of the purine riboswitch. **a** Guanine bound to the purine riboswitch (PDB ID 1Y27). **b** Adenine bound to the purine riboswitch (PDB ID 1Y26). **c** 2′-deoxyguanosine bound to the dG riboswitch (PDB ID 3DS7). The nucleotides that occupy different positions in the binding pocket are colored in *magenta*. In this and subsequent figures, magnesium ions are shown as teal spheres, water molecules as red spheres, *dashed lines* denote hydrogen bonds, and *solid lines* denote metal coordination bonds

Another single nucleotide change within the G-binding riboswitch—now at position 51—turns the selectivity in favor of dG by a factor of 12,500 (measured as the apparent dissociation constant for G over dG), principally because of an 8,400-fold decreased affinity for G. The mutation of U51 into a C allows a translation of that nucleotide so that the 2′-deoxyribose sugar attached at the N9 of dG can be accommodated in the binding pocket [73] (Fig. 2c). Similarly, high degrees of specificity and selectivity had been observed earlier in the case of an artificial RNA that had been synthesized to specifically recognize the drug theophylline **23**. This RNA was shown to have a 10,000-fold decrease in affinity for caffeine **24**, which is only different from theophylline by the presence of a methyl group at position 7 of the purine ring [74]. The NMR structure of the theophylline-RNA complex revealed an intricate network of hydrogen bonds at position 7 and in its vicinity, which would likely be perturbed by the presence of a methyl group [75]. Thus, RNA is able to scaffold specific binding sites that discriminate among closely related ligands.

Crystal structures of aminoglycosides bound to the ribosomal decoding site similarly illustrate the importance of forming direct hydrogen bonds to the RNA, particularly to adenine 1408 [28, 39]. The 6-membered sugar ring I of **6** and related aminoglycosides forms two hydrogen bonds with the Watson–Crick side of A1408 (Fig. 3a). Mutation of A1408 to a guanine is sufficient to prevent aminoglycoside binding, especially for aminoglycosides possessing a 6′-NH_3^+ group like **7** [76–78]. It was proposed that the inability of ring I to interact with a G at position 1408 was responsible for part of the absence of binding [79]. These examples jointly illustrate how the specificity and selectivity of ligand binding are tuned by finely elaborated hydrogen bonding networks. Furthermore, they emphasize the value of systematic analysis of ligand binding thermodynamics and energetics in order to properly interpret structural data, such as what was previously undertaken in the case of drug–DNA complexes [80].



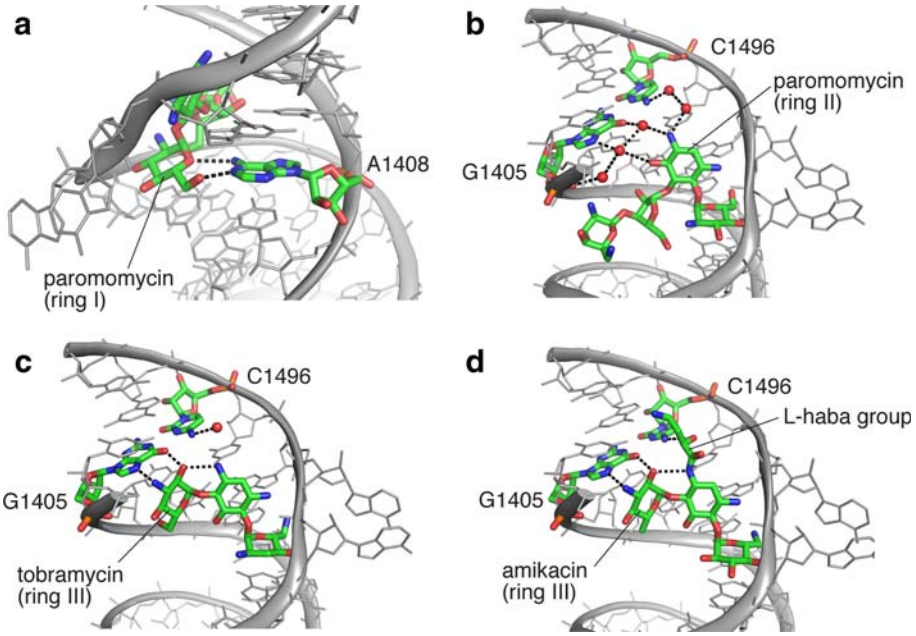


Fig. 3 Direct and water-mediated hydrogen bonds at the aminoglycoside-decoding site interface. **a** Direct H bonds to A1408 in the paromomycin complex (PDB ID 1J7T). **b** Water-mediated H bonds to the G1405=C1496 pair in the paromomycin complex (PDB 1J7T). **c** Direct H bonds to G1405 in the tobramycin complex (PDB ID 1LC4). **d** Direct H bonds to the G1405=C1496 pair in the amikacin complex (PDB ID 2G5Q)

Water to modulate ligand recognition

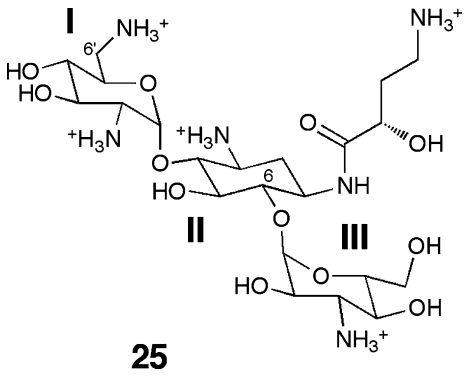
The structure of paromomycin **6** bound to the decoding site also reveals that water molecules can be an integral part of RNA-ligand recognition [39] (Fig. 3b). In fact, half of the hydrogen bonds between the RNA and paromomycin are water-mediated. This points to a main difference between the ribosomal decoding site and ligand binding sites in riboswitches: Although solvent is present at and around ligand binding sites in riboswitches, it is typically not observed to mediate ligand recognition. For example, water molecules are absent from the interface between guanine or adenine and the purine riboswitch (Fig. 2a, b) [68, 69]. Only one water molecule mediates a hydrogen bond between Lys and the RNA in a structure of the lysine riboswitch solved at 1.9 Å resolution [81]. Although several water molecules interact with dG bound to the purine riboswitch (Fig. 2c), it remains to be determined whether they would play any significant role in ligand recognition [73]. Some are, however, forming ordered networks next to the ligand of the purine riboswitch, which reflects their importance for the architecture of the binding pocket [71].

This contrast between a "wet" decoding site in rRNA and a relatively "dry" binding site in riboswitches might not be too surprising when considering the following points: (1) the decoding site is relatively open to the solvent [28, 82] compared to the interior of a riboswitch, where the ligand is >98% buried [68, 83]; (2) aminoglycosides typically possess a higher charge density than ligands of riboswitches, which favors strong interactions with water; and (3) the goal of a riboswitch is to be highly selective for a single ligand, while aminoglycosides with different chemical structures utilize the hydration properties of the RNA to recognize the same site [39, 40, 84, 85]. As an illustration of that last point, paromomycin **6** interacts with the G1405=C1496 pair via water-mediated hydrogen bonds [39] (Fig. 3b). In the complexes with tobramycin **8** and with the semi-synthetic amikacin **25** [86], these water molecules have been displaced so that hydroxyl and ammonium groups on ring III of **8** and **25**, as well as on the g-amino-a-hydroxybutyryl (L-haba) arm of **25**, form direct hydrogen bonds to the G1405=C1496 pair [84, 87] (Fig. 3c, d). Therefore, by serving as the target of diverse aminoglycosides, the ribosomal decoding site is akin to a protein-binding site that employs the properties of water to accommodate various ligands [88–90]. Recently, molecular dynamics studies have enabled a distinction between water molecules that are important for proper molecular recognition between the RNA and the antibiotic (residence time > 1.0 ns) from water molecules that could be displaced (residence time < 0.25 ns) [49]. Together, these considerations are of paramount interest for the design of modified aminoglycosides, to indicate for example where the addition of functional groups would be less likely to displace essential water molecules and compromise binding. Such reasoning may need to be adjusted for other ligands binding to other target sites, depending on their hydration properties.

Interactions involving aromatic rings

Stacking interactions: Sometimes, sometimes not

Just as π–π stacking interactions involving the aromatic bases are critical for the double helical structure of DNA



[91–94], of RNA [95–97], and for tertiary interactions that stabilize more complex RNA structures [9–11, 66], π–π stacking interactions are frequently observed between aromatic rings of ligands and RNA bases. In fact, they are responsible for the intercalation of pentamidine **5** into DNA [98] or RNA [99, 100]. More recently, the two aromatic moieties of pactamycin **10** were shown to stack on themselves, thus mimicking a dinucleotide that stacks against a G located at a tip of an RNA helix in the ribosomal exit site of the small subunit [29] (Fig. S1a). Binding to this particular site displaces part of the mRNA as it goes through the ribosome [29]. Aromatic interactions are also critical for the recognition of **1** or of an internal G by the G-binding site of group I introns during the two steps of the self-splicing reaction. By interacting with the deep groove of a Watson–Crick G=C pair [101, 102], this G forms a base triple that is sandwiched between other base triples [103–105] (Fig. S1b). Remarkably, this arrangement is identical in the structures of the three different group I introns that have been solved [105, 106], further highlighting its importance.

π–π stacking interactions are observed in riboswitch structures as well, particularly with ligands such as **19** and **20**. In both instances, the aromatic ring is stacked against purine bases on its both sides [107–110] (Fig. 4a, b). On the other hand, stacking interactions do not seem to be employed for purine recognition by the purine riboswitch [68] (Fig. 1a–c). In fact, a mutant that reverts an A–U pair

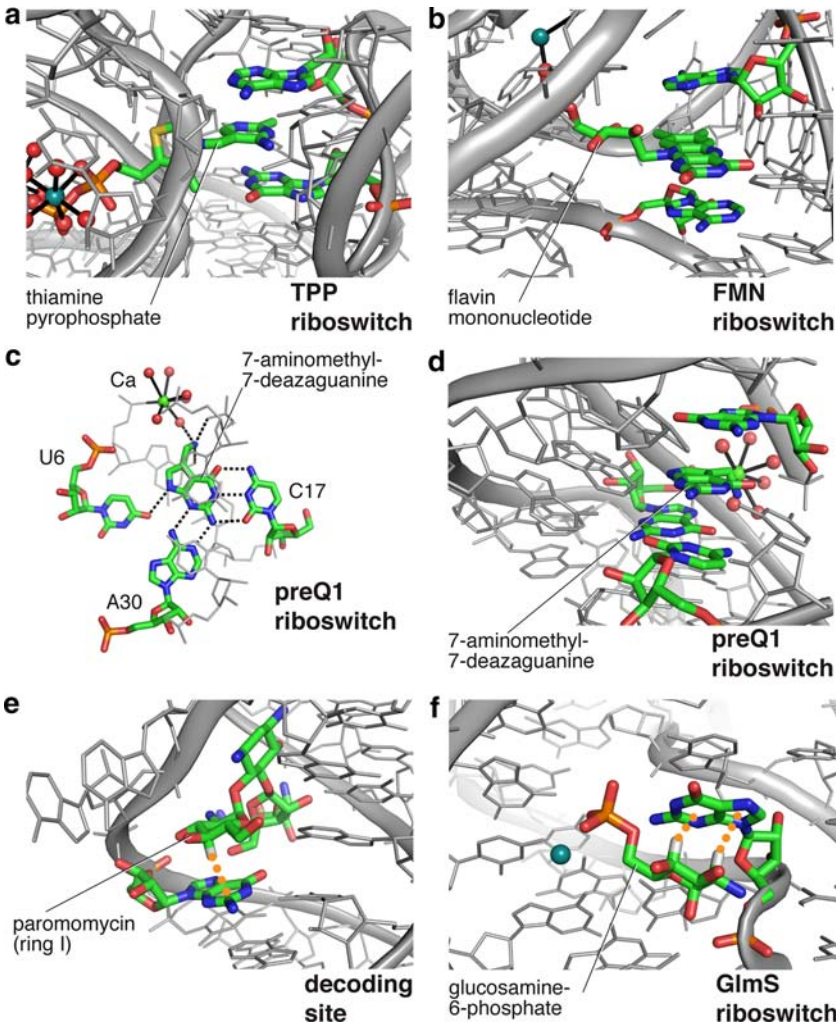


Fig. 4 Aromatic and C–H/π interactions in riboswitches and rRNA. **a** **19** stacked against a guanine and an adenine in the TPP riboswitch (PDB ID 2GDI). **b** **20** stacked against two adenines in the FMN riboswitch (PDB ID 3F2Q). **c** **17** bound to the PreQ1 riboswitch (PDB ID 3FU4). A calcium ion is shown as a green sphere. **d** Same structure as (**c**) in an orientation that shows **17** stacked against a guanine and a G=C pair. **e** C–H/π interaction (*orange dots*) involving ring I of paromomycin and G1491 (PDB ID 1J7T). **f** C–H/π interactions (*orange dots*) involving **18** and a guanine in the GlmS riboswitch (PDB ID 2Z75)

adjacent to the bound purine introduces stacking interactions without improving binding affinity [111].

Recent structures of **17** and analogues bound by the preQ1 riboswitch show that even though the minimal sizes and the global folds of the purine riboswitch (93 nucleotides organized in three helical domains brought together by loop−loop interactions) and the preQ1 riboswitch (34 nucleotides forming a pseudoknot) are different, the local organizations of the binding pocket display a striking similarity [68, 112–116] (Fig. 1a–c, 4c). In fact, the affinity of 17 for the preQ1 riboswitch and of G for the purine riboswitch are both in the same low nanomolar range [112, 113]. In addition, **17** is base pairing with a cytosine, a similar recognition mode to that of purines in the purine riboswitch [113]. However, a notable difference is the presence of stacking interactions involving **17** and bases in the binding pocket (Fig. 4d). Thereby, 17 allows continuous stacking between the two helices forming the pseudoknot [114–116]. It is therefore tantalizing to propose that stacking interactions play an essential role in the recognition of **17** by this relatively small RNA. In short, this series of examples illustrates how versatile RNA is when exploiting non-covalent interactions, from an absence of stacking (purine riboswitch) to sandwiching by purines (preQ1, FMN and TPP riboswitches).

C–H/π and cation/π interactions are also part of the picture

Other types of non-covalent interactions involving aromatic rings are also observed in structures of RNA–ligand complexes. For example, C–H/π interactions [117, 118] are formed between ring I of aminoglycosides and guanine 1491 in the decoding site [40] and between **18** and a guanine in the GlmS riboswitch [119–121] (Fig. 4e, f). These recognition modes are similar to that of carbohydrates that bind to proteins within binding sites that are lined up with aromatic amino acids (see for example [122]). In addition, a cation/π interaction stabilizes the conformation of **12** bound to the SAM-I riboswitch: The amino group at one end of the molecule points toward the adenine ring at the other end [123] (Fig. 5a). This recognition mode is similar to that occurring between a quaternary amine of acetylcholine and tyrosine or tryptophan rings from its various protein receptors [124–127]. Yet, that particular folded-over conformation of **12** is not observed in subsequent structures of **12** bound to other classes of SAM riboswitches [128, 129] (Fig. 5b), and is not often observed in SAM-bound protein structures [130–132]. Hence, its role in the recognition by SAM-I RNA remains unclear.

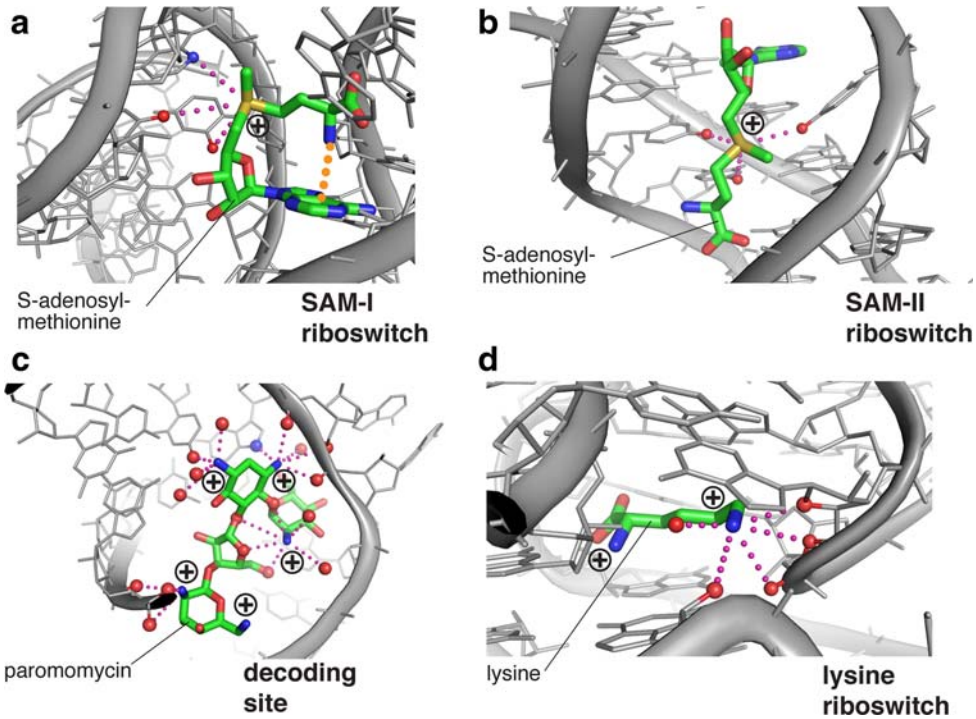


Fig. 5 Molecular recognition of positive charges by RNA. **a** **12** bound to the SAM-I riboswitch (PDB ID 2GIS). In this and subsequent panels, dipole–dipole and charge–charge interactions are shown as *magenta dots*. Here, *orange dots* denote a cation/π interaction. **b** **12** bound to the SAM-II riboswitch (PDB ID 2QWY). **c** Paromomycin bound to the bacterial ribosomal decoding site (PDB ID 1J7T). **d** Lysine bound to the lysine riboswitch (PDB ID 3D0U)

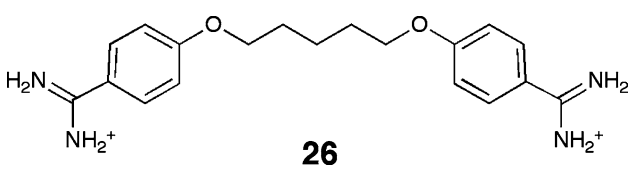
Recognition of positive charges: bring out those oxygen atoms!

Electrostatic forces are known to play an important role in folding of the RNA to a compact state [133], as well as in ligand recognition [134], probably by strengthening binding [135, 136]. For example, magnesium ions interfere with the specific binding of **8** to yeast $tRNA^{Asp}$, highlighting the importance of the antibiotic positive charges for proper RNA recognition [137]. Along that line, inhibition of self-splicing of group I introns caused by **7** or by the positively charged antimicrobial pentamidine **26** is opposed by the addition of Mg^{2+} or Ca^{2+} ions [138, 139]. Independently solved crystal structures of **6** bound to the decoding site reveal that complementary charge–charge and dipole–dipole interactions abound between the five ammonium groups of the ligand and partially charged oxygen atoms from carbonyl and hydroxyl groups, negatively charged phosphate oxygens from the RNA, and water molecules [28, 39] (Fig. 5c). Thermodynamic studies later confirmed that at physiological pH, aminoglycoside binding to the decoding site is coupled to the protonation of most of the amino groups [46].

Likewise, negatively charged environments surround the terminal amino group of Lys inside the lysine riboswitch [83] (Fig. 5d), as well as the sulfonium group of SAM inside the various classes of SAM riboswitches [123, 128, 129] (Fig. 5a, b). In the case of the SAM-I riboswitch [123], electrostatic interactions between the carbonyl groups of two uracils and the sulfonium group of **12** are proposed to account for the 100-fold selectivity for **12** over a related compound that is deprived of a charged sulfur atom [140]. Combined with binding analyses of carefully chosen chemical analogues to the cognate ligands, these structures of aminoglycoside–RNA and ligand–riboswitch complexes enable a much-anticipated advance in our perception of the specific recognition of positive charges by RNA.

Recognition of negative charges through metal coordination

The folding of RNA molecules in three dimensions is made possible in part by the binding of metal ions such as Mg^{2+}, Ca^{2+}, K^+ and Na^+ that complement the negative charges of the RNA backbone [133, 141]. Some of these metal ions



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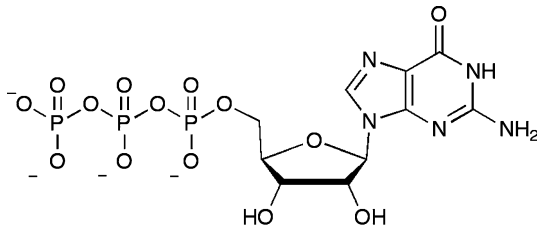

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are implicated in RNA-catalyzed reactions (see review in [142]). For example, some of the magnesium ions involved in catalysis by group I introns are bound to the G-binding site [104, 105, 143] (Fig. S1b), possibly promoting deprotonation of the attacking nucleophile [144] and stabilizing the negatively charged transition state [16, 145]. In that context, it should not come as a surprise that RNA-binding ligands possessing phosphate groups would utilize similar strategies to bind RNA. In fact, magnesium ions bridge interactions involving the phosphate groups of **18**–**20** and their respective riboswitch binding sites [108, 109, 119–121] (Fig. 4f, 6a, b). Yet, this finding was somewhat unanticipated, because the structures of artificial RNAs bound to molecules like **3** and GTP **27** suggested that metal ions are not required to achieve a specific binding with phosphorylated ligands [146, 147] (Fig. S1c). Therefore, the situation observed in natural RNAs is more akin to that in proteins, which typically require metal ions to bind **27** (see [148] and references therein) (Fig. S1d).

Considerations for drug design

Tours and detours in fathoming molecular recognition: do we ever know enough?

Even though structural and energetics studies complement each other, making sense of the results is often challenging. For example, the substitution of a hydroxyl group for an amino group at position 6′ in paramomycin **6**—leading to the chemical structure of neomycin **7**—confers to **7** an increase in binding affinity of about 10-fold at pH 7.0 [45, 149, 150]. These enhanced binding affinity is the result of a more favorable enthalpy [45]. Yet, the complete thermodynamic profiles associated with binding of these antibiotics to the decoding site reveal that binding-linked protonation is unaffected by the presence of the 6′-amino group [45]. Hence, the enhanced binding affinity could be due to a charge-dipole interaction between the antibiotic and the RNA, because the 6′-amino group is expected to be fully protonated at physiological pH [151]. Furthermore, additional hydrogen bonds formed between **7** and the RNA could account for a significant part of the more favorable

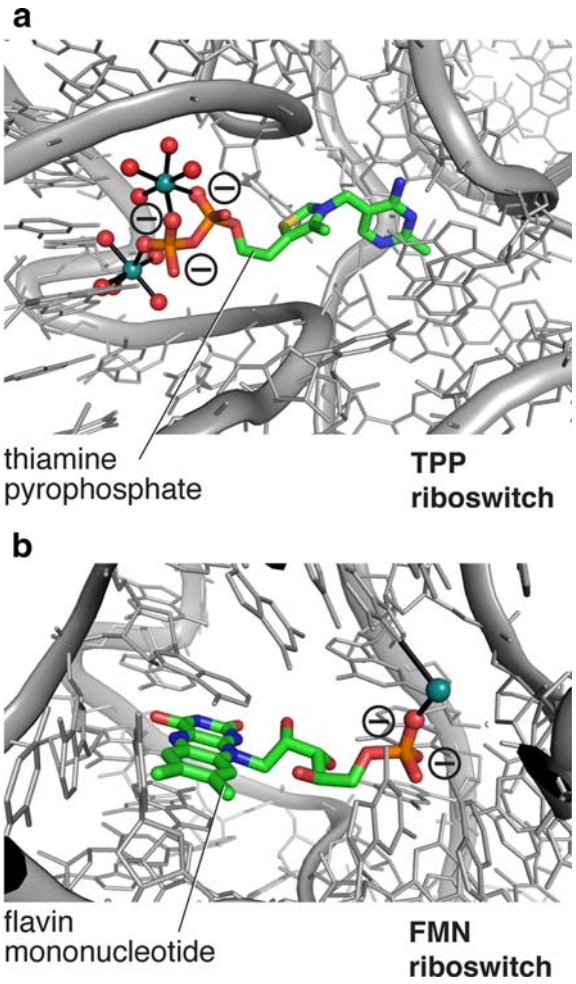
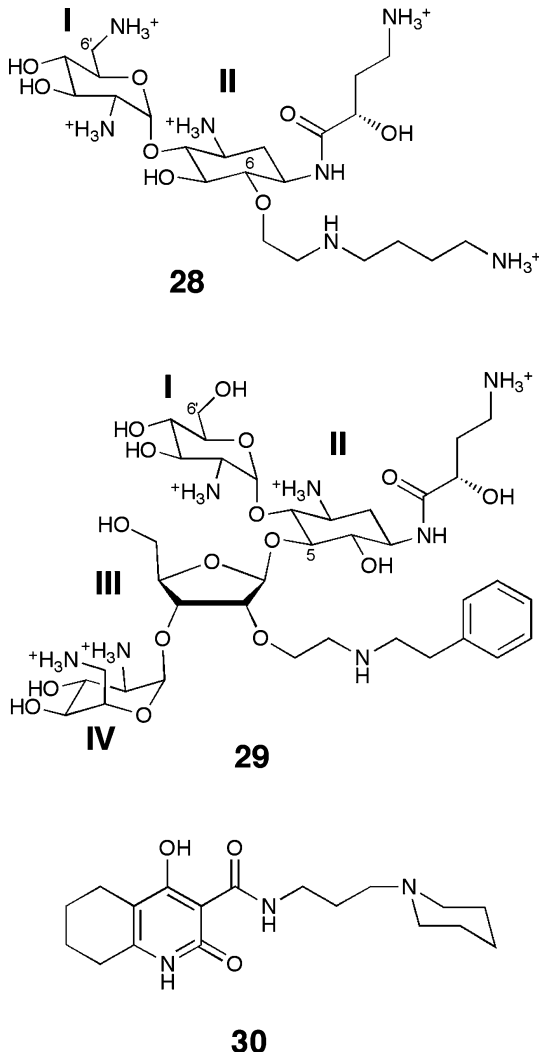


Fig. 6 Molecular recognition of negative charges by RNA through coordination to metal ions. **a 19** bound to the TPP riboswitch (PDB ID 2GDI). **b 20** bound to the FMN riboswitch (PDB ID 3F2Q)



binding enthalpy [46, 85], although this is less clear since a crystal structure of **7** bound to the decoding site was solved [42]. A third possibility involves differential hydration changes in the two complexes, as these display both enthalpic and entropic effects [45]. But here as well, subsequent results exclude that hypothesis by indicating that binding of **6** or other aminoglycosides is not accompanied by any release or uptake of water molecules [85]. Clearly, as the example above points out, the simple replacement of a functional group by another can have consequences that are not easily interpretable.

As the comprehension of such molecular recognition principles progresses, the design of novel antibiotics gets increasingly refined. Various aminoglycoside analogues have been synthesized, tested for their antibiotic activity and crystallized as a complex with the decoding site (see review in [52]). Among the most promising compounds are analogues of paromomycin **6** that contain unique basic extended arms in lieu [152, 153] (compound **28**) or in addition to some of some of the rings (compound **29**) [154–156]. These side chains are involved in additional hydrogen bonds and electrostatic interactions with the RNA that account for a superior antibiotic activity (e.g., >90% of translation inhibition at 200 mM of **28**) and an absence of susceptibility to bacterial resistance enzymes. By introducing new functionalities on the aminoglycoside scaffold, they are also responsible for enabling new specific binding sites [156]. Finally, parallel structure-based design strategies have led to the finding of potent compounds like **30** that possess original molecular scaffolds [157]. Whether any or several of these compounds will successfully pass clinical trials is presently unknown. In any case, these various examples highlight concrete efforts in expanding the antibiotic arsenal.

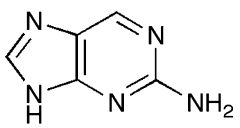
In addition, exploring molecular recognition strategies that may currently be underappreciated or that may at first seem counter-intuitive should lead to breakthroughs in drug discovery. Specifically, the analysis of halogen–oxygen (C–X···O) interactions in proteins and nucleic acids

[158] and a more recent survey of small molecules binding to protein enzymes [159] now offer guidelines on how to best take these interactions into account. Somewhat unexpectedly, anions—such as Cl^-, SO_4^{2-}, PO_4^{3-}, as well as the negatively charged carboxylic groups of aspartate and glutamate amino acids in proteins—have also been shown to possess specific recognition sites in RNA [160] (see an example in Fig. S1d). It is possible that the metal ions bound to the phosphate groups of ligands like **18**, **19** and **20** may not be critical for molecular recognition, as observed in GTP-binding proteins [148], or that they could be displaced by chemical modifications on the ligand. Finally, a recent structure of the PreQ1 riboswitch showed a Ca^{2+} ion bound 4.7 Å away from the ammonium group of **17** (Fig. 4c), reinforcing that metal ion can be located in the vicinity of positively charged groups [115]. All these properties make for promising avenues of future design strategies that would be worth exploiting.

Are folding and conformational dynamics properly taken into account?

Ultimately, small molecules destined to become drugs will be acting on ncRNAs in living environments. Hence, drug design strategies ought to take into account that RNA folds over time as it gets transcribed. In the case of riboswitches, understanding which parameters control this process may be more important than achieving a particular binding affinity [58, 161]. For example, the adenine analogue **31** happens to have a slower association rate to the purine riboswitch than adenine, despite a higher affinity [162]. Thus, adenine remains the principal effector of this riboswitch (pH 7.5 at 25 °C, 100 mM KCl, 2 mM $MgCl_2$). In this particular instance, the riboswitch is controlled by the kinetics of transcription, i.e., the transcription speed and the presence of stretches of nucleotides on which the polymerase may pause during transcription [162]. To further complicate the picture, the same riboswitch in a different genetic context could instead reach binding equilibrium before the genetic decision under its control would need to be made. In that event, binding affinity would indeed correspond to the concentration of ligand that would be required to control the riboswitch [162].

Even when considering a population of fully transcribed ncRNAs, these molecules typically exist as an ensemble of structures that contain more or less dynamic regions. Only a subset of these structures is able to bind a ligand (see for example [71, 163]). Consequently, critical insights into ligand association can be learned from studying the plasticity of the free (or unbound) states. Within the bacterial ribosome, the decoding site adopts a continuum of partially or fully open and closed conformations in the absence of ligand. The helices are mostly formed on either side of the aminoglycoside binding pocket, while the three adenines at the binding pocket adopt various flipped-in and flipped-out conformations from the helical environment [34, 41, 42, 82, 153, 164–166]. Molecular dynamics simulations reveal that the conformation corresponding to the bound state is frequently sampled in absence of ligand [50]. Therefore, ligand binding does not induce a minimal energy conformation of the RNA that can be obtained only in the presence of ligand (as what occurs during *induced fit* [167]).



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In parallel, crystallographic and fluorescence studies of the free states begin to offer insights toward aminoglycoside selectivity for the sensitive over the resistant bacterial decoding site [168], as well as for the bacterial over the human decoding site [164, 169]. In particular, the energy required for unstacking the different types of non-Watson–Crick pairs that can exist within the binding pocket in the unbound state is different in these various contexts, leading to decoding sites that possess different dynamics and that sample the conformational space differently. Since the finding that docking programs developed for proteins have a 50–60% success rate when applied to seeking RNA-binding ligands [170], RNA-centered parameters accounting for target flexibility as well as hydration have been implemented into the docking procedures [171, 172]. Further integrating such considerations about dynamics, as done for proteins [173, 174], could drastically shift the approaches of RNA ligand design [47, 171]. Such developments could help predict and validate alternative binding sites, as seen in recent studies of the complete bacterial ribosome [43] and in the crystal structure of the decoding site bound to **29** [156].

As expected, RNA dynamics vary with the ncRNA family being examined. The purine and the lysine riboswitches both offer examples of association by *induced fit*. These ncRNAs are mostly folded in absence of ligand, except for stretches of nucleotides that form the ligand binding pocket [71, 83, 175]. As in both cases the ligand is >98% buried within the RNA [68, 83], these segments are likely to serve as a port of entry for the ligand into its binding site, as described for the purine riboswitch [71, 176, 177]. The subsequent observation that these stretches of nucleotides adopt a different conformation in the structure of the purine riboswitch bound to dG [73] points toward a connection between RNA conformational flexibility and potential sites on the ligand that could be further engineered (i.e., off the N9 position of the purine ring).

Finally, ligand binding events can be correlated with larger conformational changes such as domain association. In contrast to the purine and lysine riboswitches, the SAM-I and TPP riboswitches are not globally prefolded in absence of ligand [178]. Instead, the ligand binding pocket is split between two or more domains that have different levels of preorganization. For example, the receptor of the pyrimidine moiety of TPP **19** is more preorganized than the receptor of its pyrophosphate moiety [107, 179]. Such situations are likely to occur as well for larger ligands such as coenzyme B12 **13**, as the minimal riboswitch able to recognize **13** is as large as the lysine riboswitch (~150 nucleotides), even though the molar masses of **13** (1579.5891 g mol^{-1}) and lysine **21** (146.1882 g mol^{-1}) differ by more than 10-fold [180–183]. Without the availability of all these crystal structures—that freeze-frame particular conformations—and of RNA dynamics data, chances to succeed in designing drugs that target these different ncRNAs would be significantly reduced.

Are we hitting the right target?

The value of the decoding site as a target for novel antibiotics lies in its role as the prime target for existing antibiotics, but also in its dynamic properties. Namely, the decoding site operates like a switch [28, 35, 166], whose dynamics are impeded upon aminoglycoside binding [35, 165, 184]. This and other examples illustrate the importance of targeting a molecular switch [185]. In riboswitches, the actual ligand-dependent "switching" is more intricate than first anticipated and may not even occur per se [162, 186], although ligand binding eventually induces an allosteric effect that impacts expression of the downstream gene [56, 57]. Consequently, in addition to meeting the other necessary criteria of drug targets, riboswitches operate as drugable molecular switches that influence genetic decisions.

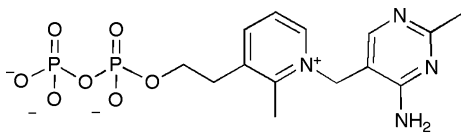
Further validation of riboswitches as a drug target came with the discovery that riboswitches are the target of some natural antimicrobials. For example, pyrithiamine pyrophosphate **32** binds with an affinity of 160 nM to a bacterial TPP riboswitch (compared to 50 nM for TPP **19** [187]) and roseoflavin **33** targets a bacterial FMN riboswitch with an affinity of 100 nM [188] (compared to 5 nM for FMN **20** [186, 189]. In both cases, antimicrobial binding to the riboswitch causes gene repression. In addition, aminopyrimidines like **34** were shown to bind to the purine riboswitch in lieu of purines [72]. Thus, it is likely that compounds could be designed that would target riboswitches, particularly if those would have original chemical scaffolds [190] that could not be broken down by the cell [62].

Nevertheless, picking the right riboswitch target is critical. For example, targeting a riboswitch that has a mostly preformed ligand binding pocket may be more attractive than targeting one that has a multi-partite pocket (see previous section). Still, recent studies show that the lysine riboswitch is not the main target of the antibiotic *S*-(2-aminoethyl)-L-cysteine (AEC, **35**) [191], although resistance mutations are principally found within this riboswitch [192, 193]. Instead, the principal cellular target of **35** is a Lysyl-tRNA-synthetase, which then employs **35** in place of Lys during translation, leading to a disruption of the protein structure [191, 194, 195]. This example is reminiscent of the finding that although the catalytic activity of a group I intron is inhibited by binding of aminoglycosides under controlled conditions outside the cell [196], this activity is indirectly suppressed inside the cell by the aminoglycoside-induced translation inhibition and an ensuing poor folding of the intron [197]. Such cases of off-target effects do not aim to rule out those ncRNAs as relevant therapeutic targets. Instead, as the example of the difference in affinity between paromomycin **6** and tobramycin **7** cited above also illustrates, they emphasize that the simplest explanation might not properly account for the observed biological effects. In the end, these at-first disconcerting findings may still lead to the design of novel antibiotics that could for instance target both the lysine riboswitch and the Lysyl-tRNA-synthetase.

Encouraging steps toward clinical approval

The tremendous expansion in our understanding of the underlying structural, energetic, kinetic, and dynamic principles that govern RNA recognition by small molecules is the fruit of concerted efforts from computational biologists, medicinal chemists, microbiologists, molecular biologists, and biochemists, both from the public and the private sectors. Small companies applied this massive amount of information to design novel scaffolds and antibiotic-hybrids that bind to known antibiotic binding sites ([157, 198, 199]), and sometimes to multiple sites simultaneously [200, 201]. As a consequence of this major advance, ribosomal RNA, riboswitches, as well as other ncRNAs and viral RNAs [202–206] are bringing back RNA on the front seat of the targets that are of major interest to the pharmaceutical industry [207]. This regain of attention from drug-development leaders should help to kick new antibiotic families through the next stages toward drug approval, including tests of bioavailability, toxicity, and effectiveness against resistant bacteria.

Fortunately, systematic efforts have been put into testing the effectiveness and the toxicity of drug candidates against a variety of resistant strains, sooner rather than later [152, 154–156, 200, 208]. For example, antibiotic-hybrid



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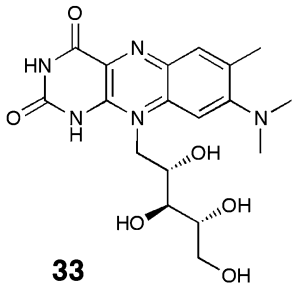

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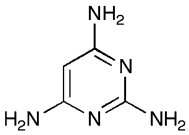

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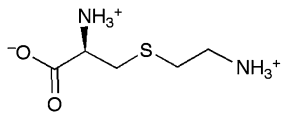

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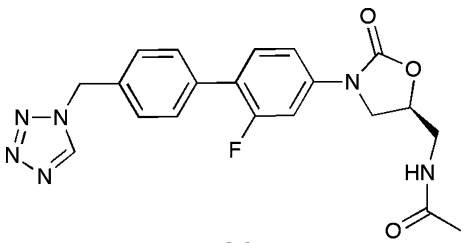

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biaryloxazolidinones like **36** synthesized by Rib-X Pharmaceuticals, Inc. (New Haven, USA) target ribosomal RNA even in multi-drug resistant bacteria like methicillin-resistant *Staphylococcus aureus* (MRSA) [209]. One of these novel antibiotics is currently undergoing Phase 2 clinical trials for skin and soft tissue infections and community acquired pneumonia (visit: http://www.rib-x.com/). Furthermore, the findings that the same aminoglycoside binds differently to the bacterial and the human decoding sites offers insights into ways of decreasing aminoglycoside-induced toxicity [210, 211]. Finally, because SAM is bound to the SAM-I riboswitch in a significantly different conformation [123] than it is bound to other SAM riboswitches [128, 129] or to most SAM-binding proteins [132], compounds could be designed that would specifically target the SAM-I riboswitch. Together, these selected examples illustrate how several compounds that target RNA could rapidly become drugs that would expand the current antibiotic battery, a foremost priority to help relieve the current resistance crisis.

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