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Perspective

# RNA-Binding Small Molecules in Drug Discovery and Delivery: An Overview from Fundamentals

Siyi Chen, Qi Mao, Hong Cheng,* and Wanyi Tai*

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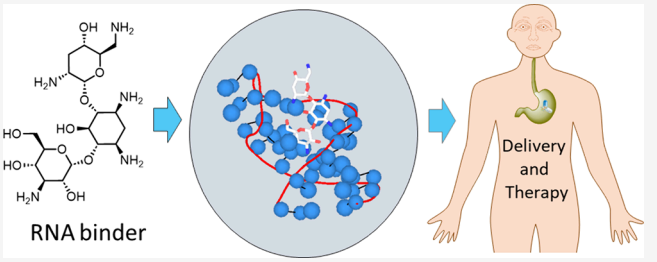
**ABSTRACT:** RNA molecules, similar to proteins, fold into complex structures to confer diverse functions in cells. The intertwining of functions with RNA structures offers a new therapeutic opportunity for small molecules to bind and manipulate disease-relevant RNA pathways, thus creating a therapeutic realm of RNA-binding small molecules. The ongoing interest in RNA targeting and subsequent screening campaigns have led to the identification of numerous compounds that can regulate RNAs from splicing, degradation to malfunctions, with therapeutic benefits for a variety of diseases. Moreover, along with the rise of RNA-based therapeutics, RNA-binding small molecules have expanded their application to the modification, regulation, and delivery of RNA drugs, leading to the burgeoning interest in this field. This Perspective overviews the emerging roles of RNA-binding small molecules in drug discovery and delivery, covering aspects from their action fundamentals to therapeutic applications, which may inspire researchers to advance the field.




## ■ SIGNIFICANCE

This Perspective explores the principles of RNA structures and their interaction modes with small molecules. It also discusses some exciting RNA-binding small molecules along with emerging conjugates such as RiboTAC for targeted RNA degradation. A focus is placed on RNA-binding small molecules in drug delivery, in which their applications in siRNA delivery and gene circuit therapy are covered. Altogether this provides a new insight to the roles of RNA-binding small molecules in drug development.

## 1. INTRODUCTION

As a critical part of the central dogma, RNA, like its partner protein, is involved in nearly all the biological processes in cells.[1] Either alone or in collaboration with other biomolecules, RNA can carry out a broad range of biological functions, from protein translation, gene regulation, and cellular differentiation to environmental response.[2] In addition to the well-known mRNA, tRNA, and rRNA which are involved in translation, noncoding RNAs account for the vast majority of the RNA sequences transcribed from the genome.[3] These noncoding RNAs, such as microRNA (miRNA), small nucleolar RNA (snoRNA), small nuclear RNA (snRNA), long noncoding RNA (lncRNA), etc., not only play vital roles in cell homeostasis but also participate in the progression of various cancers and diseases.[4] Furthermore, many viruses contain RNA as genomes.[5] These pathogenic RNA viruses, characterized by extremely high mutation rates and evolution speeds, cause outbreaks year after year, leading to countless infections and global pandemics throughout history.[6] The vital role of RNA in pathology indicates the necessity of developing drugs to manipulate the RNA functions; meanwhile, it provides numerous "druggable" targets for pharmacists to challenge diseases which previously were thought untreatable, such as Huntington's disease, neurodegenerative disease, etc.[7]

Similar to proteins, most RNAs are highly structured, 3-dimensional macromolecules. It is well known that the structure of RNA is linked to both its physical stability and biological function.[8,9] An RNA-binding small molecule, recognizing the key motif of the RNA structure, is likely able to manipulate the stability and function of RNAs.[10,11] Compared to other RNA interference methods such as antisense oligonucleotides (ASOs), siRNA, and miRNA, small molecules are gaining growing attention.[9] This might be ascribed to their advantages of oral administration, excellent permeability, and cheap production cost, particularly with extensive experience and a hundred-year history of development on small molecules against proteome.[12,13] The first RNA-binding small molecule was discovered accidentally in 1944 when streptomycin was

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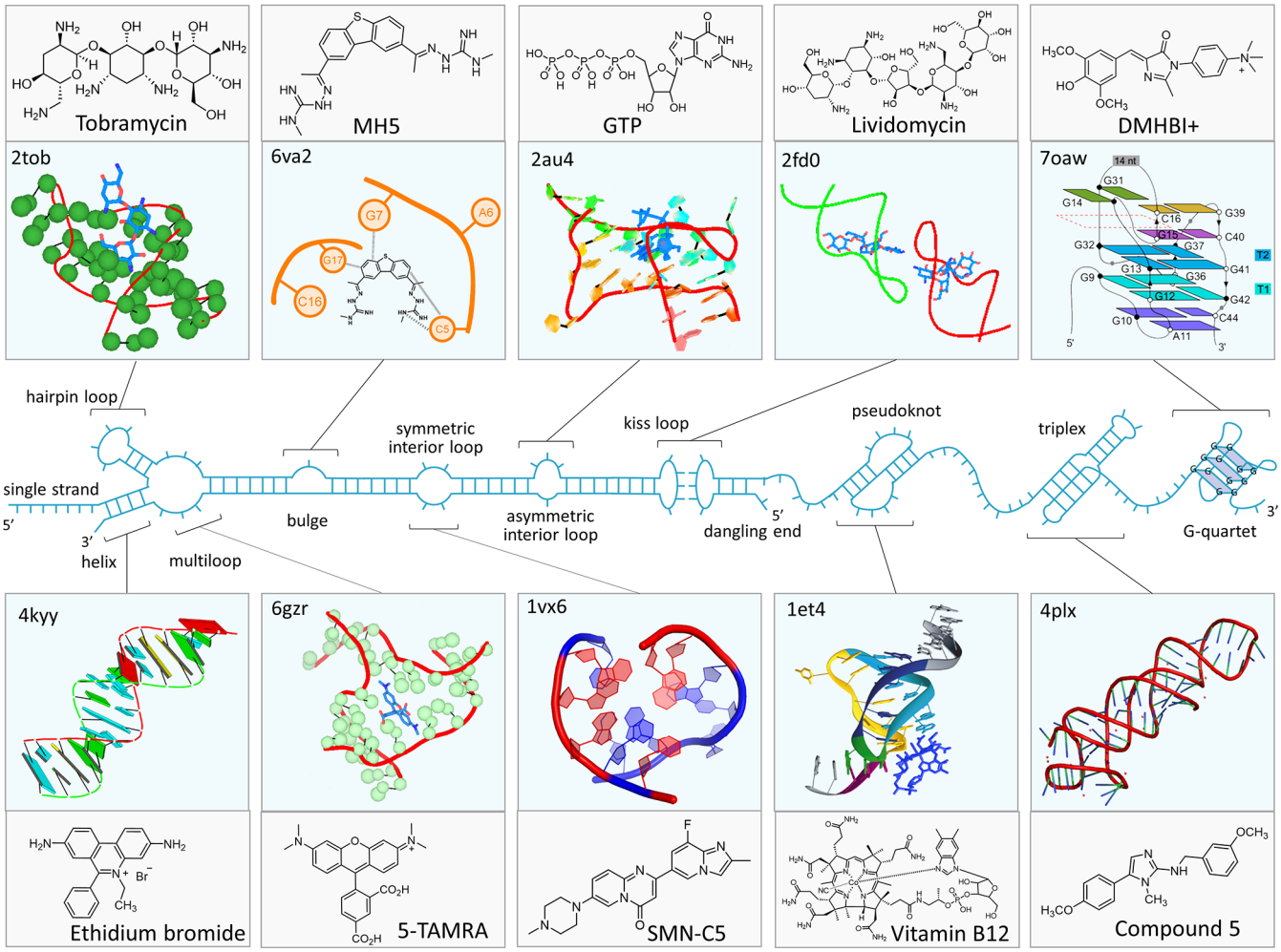


**Figure 1.** Illustration of RNA's secondary structures and their interactions with small molecules. RNA's structural elements include double helix, hairpin loop, bulge, interior loop, multiloop, kiss loop, pseudoknot, triplex, and G-quartet, all of which can afford a binding pocket to accommodate the small-molecule ligand. The examples of secondary structures in the light cyan boxes were visualized by PyMOL according to the PDB ID codes, given in the upper left corner of each panel. The chemical structures of small molecules are either highlighted in blue with RNA or displayed planarly in the light gray boxes.

identified as an antibiotic from the bacteria *Streptomyces griseus*.[14] Its mode of action was later revealed to be related to the translation-stalling toxicity caused by binding with the bacterial ribosomal RNA (rRNA).[15] Subsequently, other natural products, such as macrolides, tetracycline, and oxazolidinones, have been identified as rRNA binders with antibiotic properties as well.[16] The therapeutic paradigm of RNA-binding small molecules has been further expanded to other targets and indications recently, such as the FDA-approved Risdiplam for spinal muscular atrophy (SMA) and Zotatifin in a clinical trial for metastatic breast cancer.[17,18] The increasing interest in RNA targets has led to many compounds being documented as RNA-binding small molecules.[19,20] They are reported to be able to target landscapes across a broad range of RNA types, including miRNAs, RNA splicing sites, RNA expansion elements, riboswitches, viral RNA motifs, etc.[21,22]

Given this premise, small molecules might become one type of important therapeutics against RNA targets in the future, following the dogma of chemical drugs employed for enzymes and receptors.[23,24] Meanwhile, it is worth noting that the disease-relevant RNAs, in contrast to most protein targets, exist in dynamic structures which can vary depending on base-pairing patterns and environmental factors.[25,26] In this regard, there is ongoing appreciation that therapeutics based on RNA-binding small molecules might be more difficult to develop than those involving protein binders.[10] In this Perspective, we illustrate the interaction modes of small molecules with the RNA target and discuss several druggable RNA motifs which are currently accessible by small-molecule binders. The most promising therapeutic candidates for RNA-binding small molecules are also classified and summarized according to their action modes. Finally, a focus is placed on the emergent modalities adding new functions to the specific RNA binders, such as RNA-targeted degrader compounds, RNA-binder-based delivery systems, and synthetic RNA circuits controlled by RNA-binding small molecules. This Perspective provides a general overview of RNA-binding small molecules in drug discovery and delivery, which may lay out a roadmap for pharmaceutical scientists to enter this emerging field.

## 2. SMALL-MOLECULE RECOGNITION OF RNA STRUCTURE

Different from proteins that have a total of 20 natural amino acids, RNA consists of only four primary nucleotide units (A, U,


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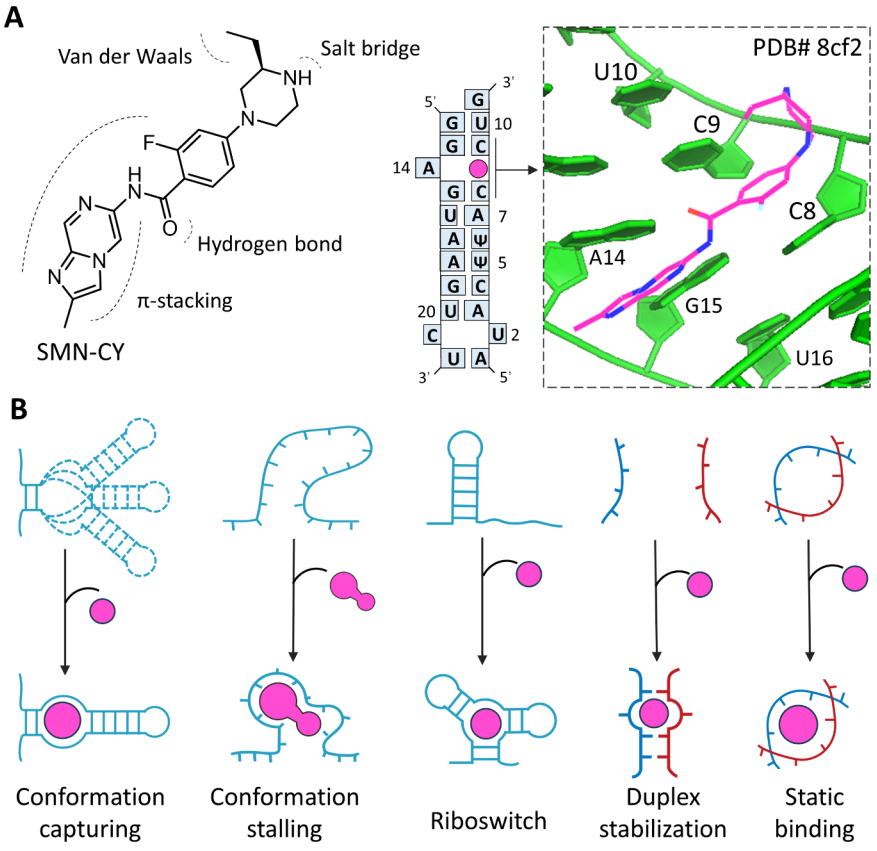


**Figure 2.** Binding modes of small molecules with RNA. (**A**) The complex of compound SMN-CY and RNA bulges by $\pi$-stacking, hydrogen bond, salt bridge, and van der Waals forces. The NMR structure of the bulge bearing the small-molecule ligand is shown on the right (PDB ID: 8cf2). Ψ is the modified RNA base pseudouridine. (**B**) Recognition modes of small molecules toward the dynamic RNA. Conformational adaptation of RNA accounts for an equilibrium of energy, one of which allows sufficiently strong molecular interactions. The filled pink cycles represent the RNA-binding small molecules.

G and C) as building blocks. It seems less variable than proteins that contain high chemical diversity, e.g., basic, acidic, hydrophobic, hydrophilic, and charged properties of amino acids. However, the four nucleotides can form different types of base−base interactions covering a geometric base-pairing family of 12 members.[27] The base-pairing patterns dictate the formation of elaborate RNA structures and control the essential functions of RNA in biology. The highly structured RNAs, similar to proteins, tend to contain pockets permitting the specific binding of small molecules.

**2.1. RNA Structure and Its Small-Molecule Ligands.** Similar to protein, the complicated tridimensional architecture of RNA can be referred to as primary, secondary, and tertiary structures, respectively. The primary structure is the RNA sequence composed of four ribonucleotide bases. The base pairs associated by these bases define the secondary structure of RNA.[28] The most common base-pairing interaction forms through the canonical Watson−Crick pattern, where the base pairs consist of A-U and G-C only. Many other noncanonical base pairs have also been discovered, such as Wobble base pairing (G·U pairs), Hoogsteen base pairing, etc., all of which can be classified into the 12-membered base-pairing family based on their base orientation and stacking modes.[28] The diverse patterns of base pairing enable the formation of many distinct structures that drive biological processes in cells.

The perfect match of base pairs in RNA allows the formation of a duplex or helix. It is worth noting that the RNA duplex tends to form a wider A-form conformation, which is distinct from the B-form of DNA.[29] In addition to the duplex regions, nucleotides that have not formed pairs generate single-stranded structures. By a combination of duplex and single strands, RNA adopts more complicated motifs, including hairpin, bulge, symmetric interior loop, asymmetric interior loop, and multiloop (Figure 1). When two hairpins are brought in proximity and form a base−base interaction at the loop region, a kiss loop forms. If the two hairpins are partially intertwined, they can fold into another important motif, the pseudoknot (Figure 1). This motif has been discovered in many viral genomes and plays a role in the regulation of gene expression.[30] Triplexes, G-quartets, and tetraloops are also secondary motifs commonly seen in RNA structures.[5] Small molecules have the potential to directly bind with the secondary RNA motifs by interacting with a region of particular base groups and electrostatic surface, similar to molecular recognition in protein pockets.[10] Figure 1 shows 10 examples of small-molecule ligands interacting with various RNA motifs, including ethidium bromide,[31] Tobramycin,[32] MH5,[33] GTP,[34] Lividomycin,[35] DMHBI+,[36] 5-TAMRA,[37] SMN-C5,[38] Vitamin B12[39] and compound 5.[40] A full list of RNA-binding small molecules is reviewed and detailed elsewhere.[10,20] It is worth noting that the bulge motif seems more likely to generate pockets to accommodate small molecules, as one-third of the reported RNA-binding small molecules were identified to bind with bulge motifs.[13] Interactions between small molecules and secondary structures might lead to confirmational changes, gene regulation, function manipulation, or biological responses, which provide a means to treat diseases.


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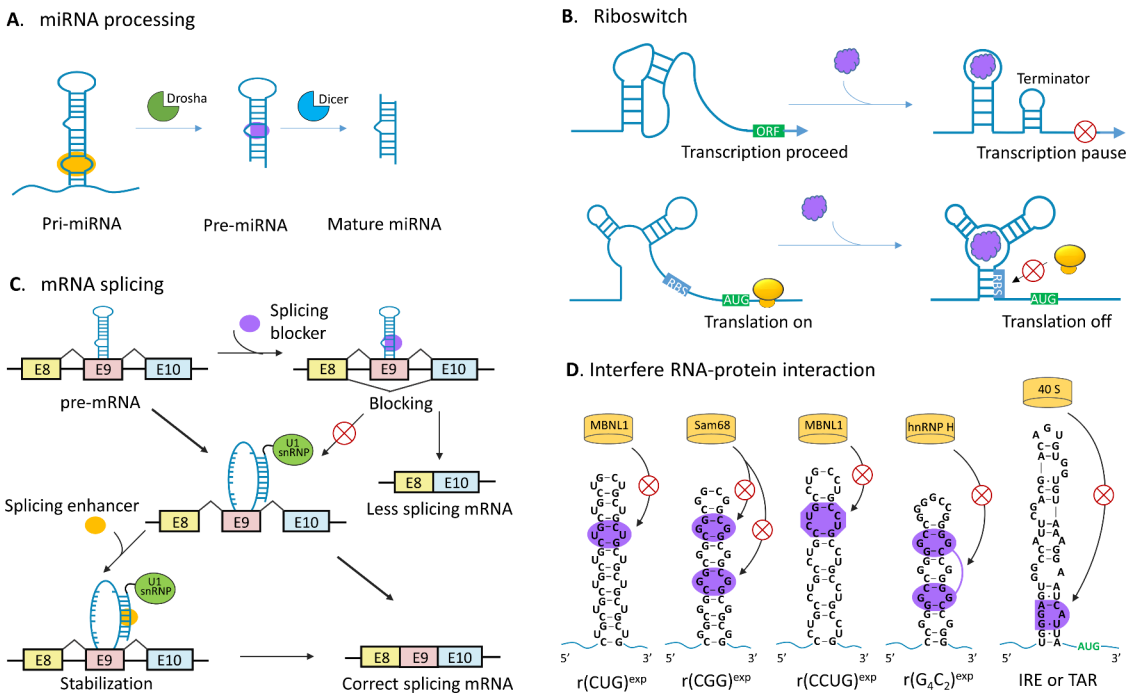


**Figure 3.** Druggable RNA motifs. (**A**) Small molecules block the Drosha binding site (yellow) or Dicer binding site (purple) to prevent miRNA processing and maturation, thereby regulating the miRNA pathway. (**B**) Binding of the small-molecule ligand to the riboswitch at the 5′ UTR region of mRNA induces a conformational change that prematurely aborts the transcription or translation. (**C**) Small molecules modulate the mRNA splicing pattern by either blocking U1 snRNP assembly with pre-mRNA or enhancing the assembly stability. (**D**) Small molecules targeting r(CUG)$^{exp}$, r(CGG)$^{exp}$, r(CCUG)$^{exp}$, or r(G4C2)$^{exp}$ can release the sequestered RNA-binding proteins and rescue their biological functions, thus slowing down the disease progress. Small molecules can also inhibit the translation of traditionally undruggable proteins by targeting the IRE or TAR region of their encoding mRNA. The filled purple cycles represent the RNA-binding small molecules.

The association of RNA secondary structures can further increase the structural complexity, defined as RNA tertiary structures. The formation of tertiary organization is mediated by various intramolecular interactions such as base stacking, van der Waals interactions, and hydrogen bond formations. If tertiary RNA assembles with other biomolecules, for example, DNA, proteins, or other RNAs,[21] these high-order RNA complexes are referred to as quaternary structures. Tertiary and quaternary RNA represent the most complicated biomolecules in cell biology, and their structural determination—even computational prediction—remains an ongoing challenge.[41] Nevertheless, the complicated assembly provides a basis to develop high-quality RNA-binding small molecules because the pockets formed by these RNAs are generally unique, special, and structurally distinct from other RNA molecules. This rationale was bolstered by the success of some RNA-binding small molecules in the clinic, for example, the FDA-approved aminoglycosides that recognize bacterial rRNA (tertiary structure) and zotatifin, in clinic trials, that interrupts mRNA/eIF4A ribonucleoprotein assembly (quaternary structure). Both of these are discussed later in detail.

**2.2. Binding Modes of Small Molecules with RNA.** With the increasing number of studies of RNA-binding small molecules, it is becoming clear that small molecules recognize RNA motifs in a manner different from the proteins. The difference lies in at least two aspects. The first, in this regard, is the type of interaction forces between small molecules and biomacromolecules. A comparative analysis of RNA/small molecule complexes and protein/small molecule complexes by the Schneekloth group revealed that the major contributors to RNA recognition are $\pi$-stacking forces and hydrogen-bond interactions.[42] As a result, the RNA-binding small molecule generally contains, but not always, at least one extended aromatic ring system, a key feature distinguishing it from the protein binders. It is speculated that these aromatic rings can insert themselves between the stacked base pairs and strengthen their interaction (Figure 2A).[33] Hydrogen-bonding interactions, especially salt bridges (between small-molecule amines and RNA phosphates), also occur between small molecules and nucleotides. Conversely, the hydrophobic contact that dominates protein/small molecule recognition is under-represented in RNA/small molecule binding mode.[23] It is probable that the negatively charged phosphate backbone of RNA dislikes access by hydrophobic small molecules but favors interactions with positively charged species. As can be seen in the examples of Figure 1, most compounds contain either amine, guanidine, or another chemical group that can protonate at physiological pH and provide ionic hydrogen-bond interactions with the RNA phosphate backbone. Nevertheless, hydrophobic interactions cannot be neglected, even though they occurs less frequently in RNA/small molecule recognition. The ethyl group of SMN-CY in Figure 2A appears to form van der Waals interactions with RNA bases, probably increasing the affinity.[43]

Second, the RNA targets of small molecules are structurally more flexible than the protein targets. RNA is a dynamic assembly of substructures, changing with the base-pairing pattern, intermolecular interactions, ionic conditions, and environmental stimuli.[25] The engagement of small molecules into RNA alters the conformational landscape and may impact the functions of RNA.[26] The well-known RNA/small molecule binding modes include conformation capturing, conformation stalling, riboswitch, duplex stabilization, and static binding, as


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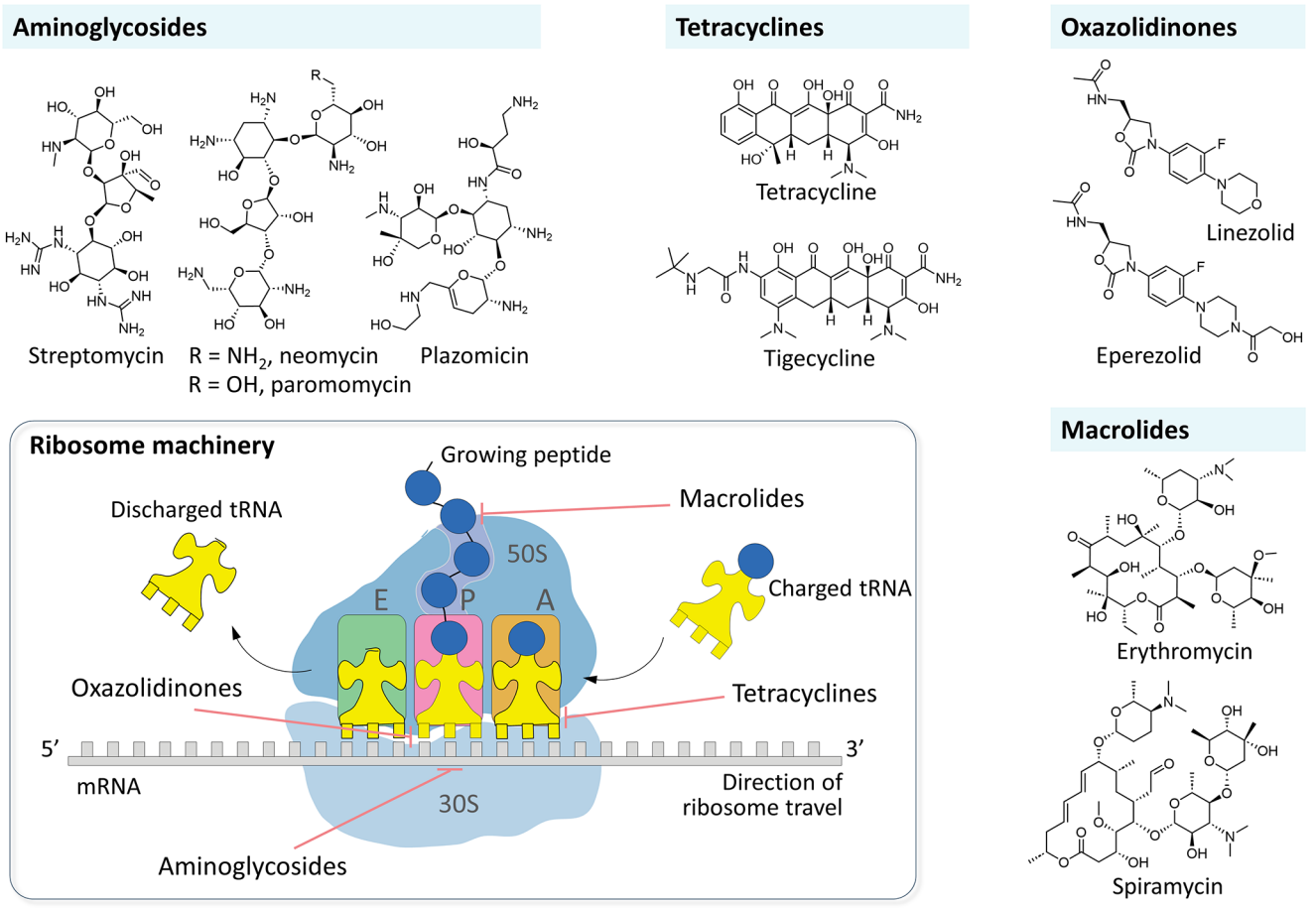


**Figure 4.** Chemical structures of the RNA-binding antibiotics that block the ribosome machinery. The antibiotics bind with various sites of rRNAs and inhibit peptide elongation. The mechanism of ribosome machinery and the action sites of antibiotics are depicted in the light gray box.

shown in Figure 2B. This diversity in action modes offers flexible ways for RNA recognition and function modulation by small molecules.[10] For example, Ribocil, an antibacterial that targets the flavin mononucleotide (FMN) riboswitch, captures the RNA conformation and represses the riboswitch-mediated ribB gene expression, which eventually leads to the inhibition of bacterial growth.[44] The conformational dynamics of RNA, on the other hand, raise a challenge to the design and discovery of RNA-binding small-molecule drugs. The Inforna strategy, Chem-CLIP screening, and AI-assisted rational design approaches have been created to tackle this challenge. Several excellent reviews on RNA-binding small molecule identification have covered this topic.[10,13,21] Besides structural dynamics, it is worth noting that RNA can be post-transcriptionally modified.[45] Modifications of RNA, such as base methylation, can alter the RNA folding and 3D structure (a phenomenon termed the m6A switch),[46] which increases the difficulty to identify small-molecule binders for an RNA target.

**2.3. RNA Targets Druggable by Small Molecules.** Recent studies estimate that up to 85% of the human genome can be pervasively transcribed into RNA, which results in a plethora of RNA products over 84 gigabases in every human cell.[47] Around 90% of all transcriptional output in humans is the noncoding RNAs.[48] Some small-molecule-binding pockets have been identified in these RNAs, for example, the miRNA processing site, the RNA splicing regulatory element, the IRES site of viral RNA, etc., which enable small-molecule accommodation and thus tune the RNA's biological functions. Here we describe several representative RNA targets for small molecules. A more comprehensive summary is available in reviews elsewhere.[7]

miRNA is a class of small noncoding RNAs that function in post-transcriptional gene regulation. miRNA is initially transcribed as primary miRNA (pri-miRNA) and then digested by the enzyme Drosha to generate precursor miRNA (pre-miRNA).[49] pre-miRNA is subsequently processed by Dicer to yield a mature miRNA 21−25 nucleotides in length. Dysregulation of miRNA is implicated in a variety of human diseases, including cancers.[50] Small molecules that block miRNA processing, for example, occupying the Drosha or Dicer binding sites, can slow down the miRNA processing and modulate the disease's progress (Figure 3A). Riboswitch RNA is another valuable target for small-molecule recognition.[26,51] Riboswitch is a gene-control structure commonly found in the 5′ untranslated regions (UTRs) of mRNAs.[52] This motif responds to a small-molecule ligand and undergoes a folding pattern change, which regulates the downstream gene expression by the transcription terminator or RBS sequestration (Figure 3B).[53] The development of the antibacterial small molecule Ribocil validates this type of RNA target and elucidates the therapeutic potential.[44]

Eukaryotic pre-mRNA processing is another cellular event that can be targeted by RNA-binding small molecules. Almost every splicing step requires the assemblage of various regulatory


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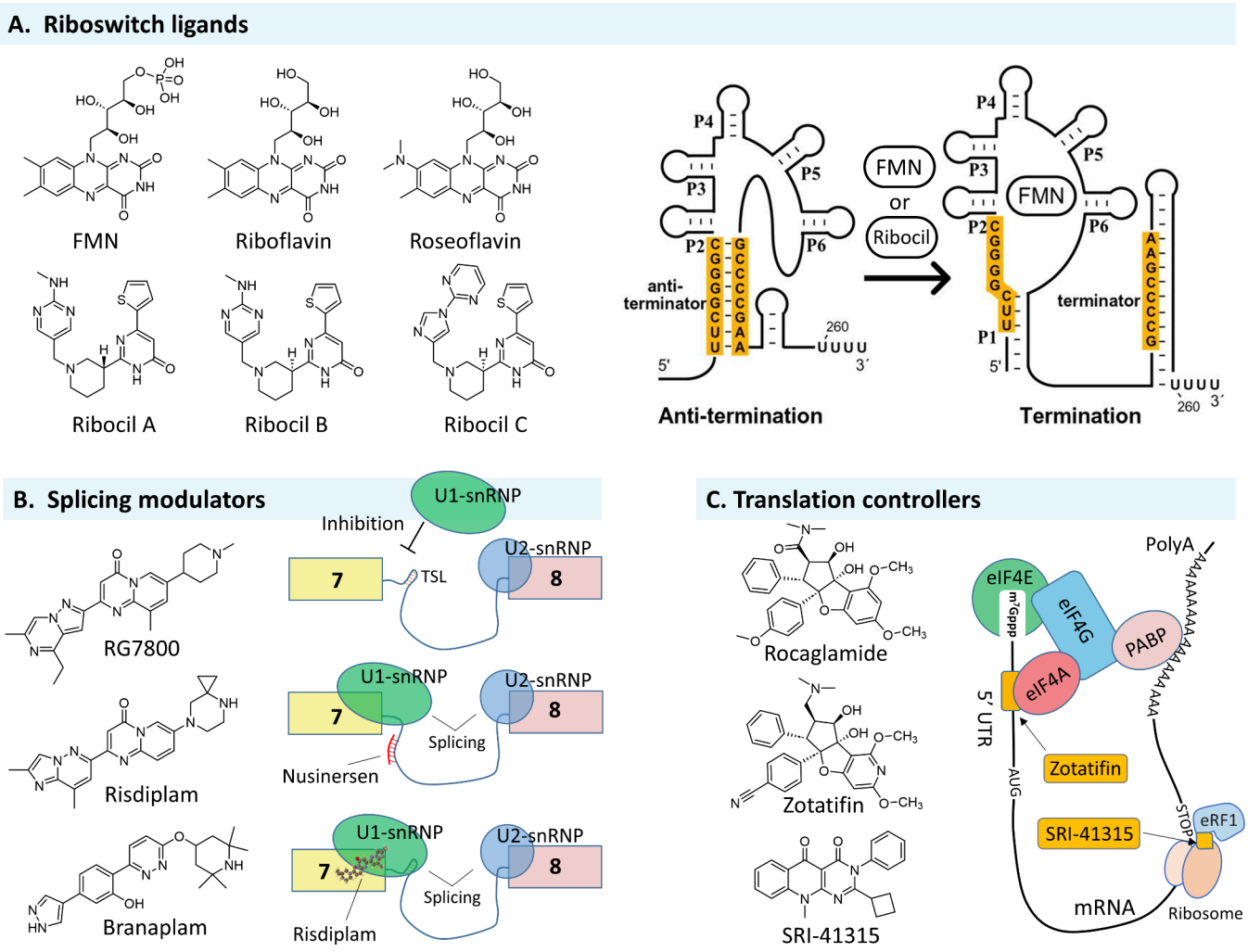


**Figure 5.** Chemical structures of Ribocil, Risdiplam, Branaplam, and Zotatifin and their analogs. Their mechanisms of action are depicted to the right of the chemical structures. (**A**) Riboswitch ligands Ribocil A, B, and C and their natural analogs. FMN or Ribocil mediates a riboswitch that unmasks the putative terminator structure (shaded regions) and terminates the downstream transcription. Adapted with permission from ref 68. Copyright 2002 National Academy of Sciences. (**B**) The terminal stem-loop (TSL) structure formed at the end of SMN2 exon 7 contributes to exon 7 skipping by inhibiting the association of U1-snRNP with the 5′ splice site. Antisense oligonucleotide Nurinersen disrupts the TSL and promotes the access of U1-snRNP, while splicing modulator Risdiplam enhances the binding of U1-snRNP to the splicing site by glutting these two components. (**C**) Anticancer drug candidate Zotatifin is derived from natural product Rocaglamide and acts as a complex glue to stabilize the incompetent RNA/eIF4A complex, leading to oncogene translation repression.

proteins (such as U1 snRNP, and splicing factor proteins) to promote effective splicing.[54] Splicing site mutation can alter splicing patterns and thus truncate the encoded protein, which causes human diseases.[7] Overwhelming evidence has demonstrated that it is possible to rescue the splicing defects by stabilizing the mispaired spliceosomal machinery with RNA-binding small molecules.[55] Inversely, exon exclusion can also be promoted by occluding the splicing regulatory element (SRE) from spliceosomal assembly by small-molecule binders (Figure 3C). Some small molecules targeting pre-mRNA splicing have been developed for SMA, for example, Risdiplam and Branaplam, both of which are covered in the next section. RNA/protein complexes, namely ribonucleoprotein assemblies, are one type of quaternary RNA involved in various biological processes, and their malfunction underlies the origin of many diseases.[56] Destabilizing the RNA−protein interaction is the most important strategy of RNA-binding small molecules in therapeutic applications.[57] The targetable RNA motifs of small molecules include but are not limited to the RNA repeat expansions, such as $r(CUG)^{exp}$, $r(CGG)^{exp}$, $r(CCUG)^{exp}$, and $r(G_4C_2)^{exp}$ that are responsible for over 30 human diseases, and IRE or TAR motifs that regulate mRNA translation machinery (Figure 3D). This topic has been extensively reviewed by Meyer et al.[7] It is worth noting that the protein component in these ribonucleoprotein targets is generally considered "undruggable" due to the lack of small-molecule binding pockets. RNA-binding small molecules provide an alternative route to manipulate the biological functions of these ribonucleoproteins, reflecting the therapeutic value of RNA targets.

## 3. RNA-BINDING SMALL MOLECULES AS THERAPEUTICS

Several small molecules that target RNAs have demonstrated therapeutic values. They can be classified as below.

**3.1. Ribosome Binders: Aminoglycosides, Tetracyclines, Oxazolidinones, and Macrolides as Antibiotics.** Ribosome is a primary component of cells that is involved in protein synthesis. It is a large ribonucleoprotein complex


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consisting of proteins and rRNA, with rRNA dominating the main functional sites. The bacterial ribosome 70S, assembled by two smaller subunits (50S and 30S), is composed of three rRNAs (5S, 23S, 16S) and ∼52 ribosomal proteins.[58] The rRNA ensures the proper alignment of the mRNA and tRNA with the ribosomes and also catalyzes the peptide bond formation during protein synthesis.[16] Ribosome-targeting antibiotics act by interacting directly with rRNA and inhibiting the translation function (Figure 4).[59] For example, aminoglycosides are known as antibiotics that target the decoding region of 16S rRNA and induce miscoding errors during protein synthesis.[60] This class of antibiotics is comprised of both natural products (such as Streptomycin, Neomycin, and Paromomycin) and semisynthetic derivatives (such as Plazomicin).[19] They share a similar mechanism of action and possess a broad spectrum of activity against Gram-negative and Gram-positive organisms, including those that are multi-drug-resistant. The antibiotic tetracyclines are believed to inhibit bacterial translation by binding to the 16S rRNA. Tetracyclines sterically block the access of aminoacyl-tRNA to the mRNA−ribosome complex, leading to a translation halt.[61] Meanwhile, macrolides (e.g., Erythromycin, Spiramycin, etc.) can bind to the 23S rRNA in the nascent peptide exit tunnel and inhibit protein synthesis.[62] Some synthetic antibiotics have also been found to target specific sites on ribosomes. For instance, Linezolid and Eperezolid inhibit the translation of bacterial ribosomes by binding to the peptidyl transferase center in 23S rRNA.[63] It is interesting to note that synthetic antibiotics are designed to contain chargeable amine groups that can interact with the negatively charged RNA backbone; this chemical feature is also shared by natural ribosome binders.

**3.2. Riboswitch Ligand: Ribocil.** Riboswitch is a common RNA motif in the 5′ UTR of mRNA that controls the translation of genes.[64] In bacteria, riboswitches are specifically responsive to endogenous metabolite ligands, such as amino acids, vitamins, and FMN, for gene expression regulation.[65] Synthetic small molecules may be designed to mimic the action of these ligands and govern the expression of target genes.[66] For example, Roseoflavin, a chemical analog of FMN and riboflavin, was designed to bind with bacterial FMN riboswitch, showing antibacterial activity (Figure 5A).[67,68] A phenotypic screening by Merck reported another structurally distinct compound, Ribocil, that targets FMN riboswitch with a $K_D$ value of 25.6 nM.[44] The lead compound Ribocil is a racemic mixture of isomers Ribocil A (*R*-enantiomer) and Ribocil B (*S*-enantiomer), with Ribocil B found to be responsible for the bioactivity.[69] A further structure−activity relationship (SAR) study discovered Ribocil C as the most potent riboswitch ligand ($K_D$ < 1 nM),[70] exhibiting a broad spectrum of antimicrobial activities.[71,72] A crystal structure study revealed that Ribocil competitively binds to a riboswitch site distinct from the endogenous FMN site.[73] The development of Ribocil was halted in the preclinical stage due to the emergence of bacterial resistance,[72] but this finding enlightens a milestone of the RNA-binding small molecules to target dynamic RNAs.

**3.3. Splicing Modulators: Risdiplam and Branaplam.** SMA is a genetic disease caused by the survival motor neuron (SMN) 1 gene mutation, with a subsequent change of the pre-mRNA splicing pattern and decreased expression levels of SMN protein in the spinal cord.[74] Promotion of the SMN2 splicing into a full-length mRNA was proven to be an effective therapeutic strategy, as demonstrated by the FDA-approved antisense oligonucleotide drug Nusinersen (Figure 5B).[17,75] The orally bioavailable small molecule RG7800 was identified to bind with pre-mRNA of SMN2 and can modulate the splicing pattern.[76] NMR spectroscopy revealed that the compound binds with an adenosine bulge at the exon 7−intron junction, acting as a stabilizer for the U1-snRNP/pre-mRNA complex.[38,77] Stabilization of the spliceosome complex promotes the exon inclusion and yields a splicing of full-length mRNA.[78] The subsequent SAR study around RG7800 led to Risdiplam, the first RNA-binding small molecule approved by the FDA to treat SMA.[79] Branaplam represents another small molecule that modulates the SMN2 pre-mRNA splicing pattern.[80] A series of studies pinpointed that Branaplam enhances the splicing of full-length SMN2 mRNA in a way similar to that of Bisdiplam but interacts with a GA sequence at the end of exon 7 and acts as a molecular glue to stabilize the transient dsRNA structure formed by U1 snRNP and SMN2 exon 7.[81] Branaplam was evaluated in clinical trials for the treatment of SMA (Phase 1/2) and Huntington's disease (Phase 2), but the trials were discontinued by Novartis due to the symptoms of nerve damage.[82,83]

**3.4. Translation Controller: Zotatifin.** Translational control is an important strategy of cancer cells to regulate oncogene expression and promote tumorigenesis. Its regulation by cancer cells is multifaceted, involving non-sense mutation, translation factor overexpression, and alterations in the translation-associated signaling pathway.[84] The UTR regions, including 5′ and 3′ UTR of mRNAs, determine the intrinsic translational efficiency and are involved in these processes.[85] Studies have shown that some sequence and structural motifs in 5′ UTRs, serving as structural regulatory elements, tend to be overrepresented in oncogenic mRNAs, conferring the tight regulation of their translation in normal cells.[86] These featured RNA motifs, for example, the polypurine motifs, have been found in many oncogenes and survival factors, including receptor tyrosine kinases (RTKs), KRAS, Cyclin D, CDK4/6, and MYC.[87,88] Zotatifin (eFT226) is an RNA-binding small molecule derived from the natural product Rocaglamide.[89−91] Zotatifin elicits a potent antitumor activity by inhibiting the translation of oncogenic mRNAs that contain polypurine motifs at the 5′-UTRs (Figure 5C).[92,93] It downregulates the oncoprotein translation and demonstrates potent antitumor activity across a broad range of solid tumor models.[94,95] A molecular modeling study revealed that Zotatifin can bind with eIF4A and RNA motifs simultaneously via the van der Waals and $\pi$-stacking forces.[89] It acts as an RNA−protein interface glue and blocks the 43S preinitiation scanning of start codon AUG, resulting in selective translational repression.[93] Zotatifin represents a typical example of RNA-binding small molecules that interfere with the RNA quaternary structure, validating the broad therapeutic potential of RNA-targeting molecules. Zotatifin was currently evaluated in a Phase 2a clinical trial as a monotherapy or combination treatment for breast cancer and non-small-cell lung cancer.[18] SRI-41315 is another molecular glue that can stabilize the eukaryotic ribosome with eRF1 (an essential translation termination factor that recognizes stop codons). The complex stabilization by SRI-41315 will finally trigger eRF1 degradation and subsequent termination of translation at near-cognate stop codons.[96] SRI-41315 is a lead screened from 771,345 compounds. Its special action model provides a promise for non-sense suppression therapy.[97]

## 4. TARGETED RNA DEGRADATION BY BIFUNCTIONAL RNA-BINDING SMALL MOLECULES

Targeted degradation by small molecules was first demonstrated on protein targets. It emerged as a novel pharmaceutical


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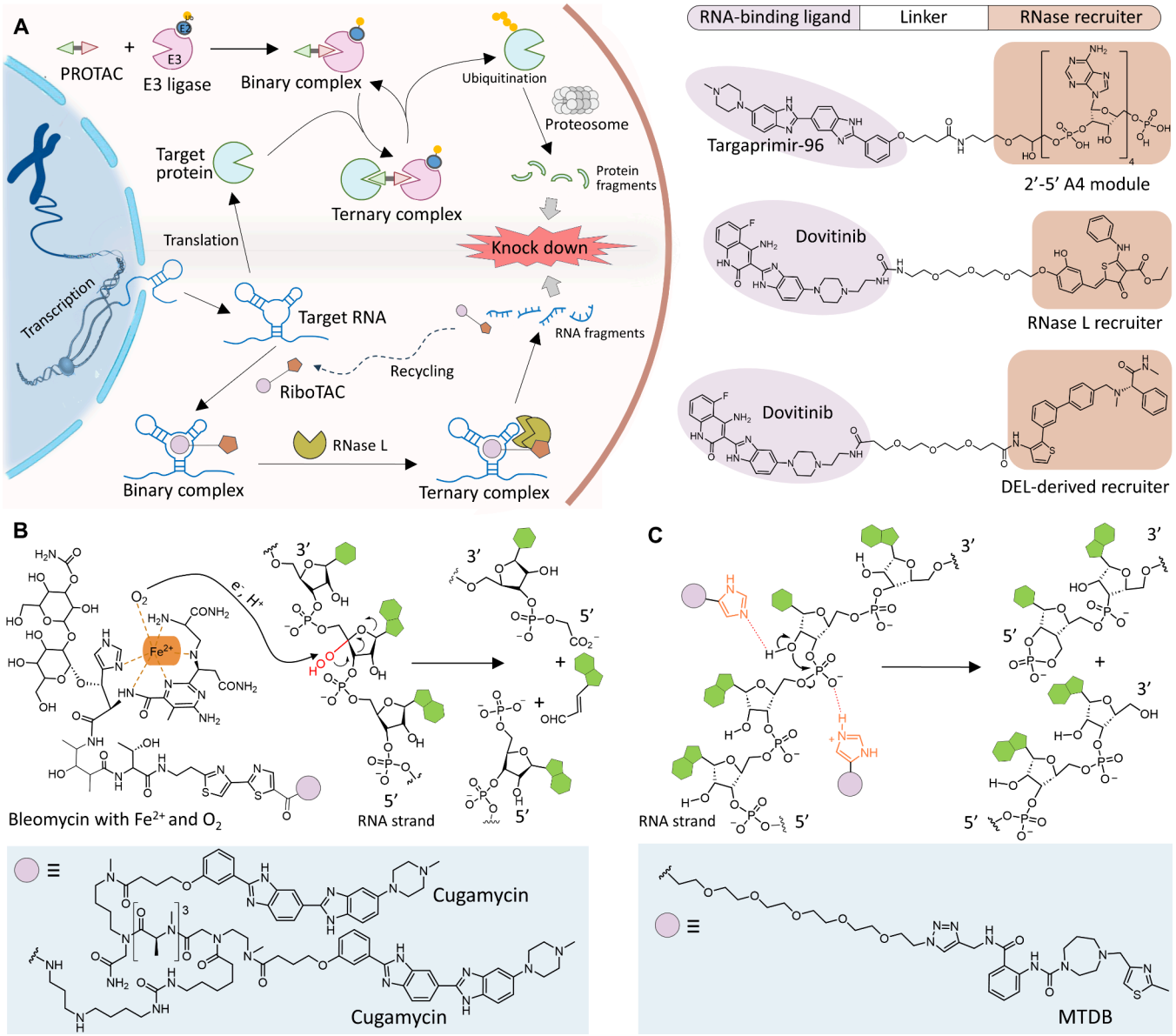


**Figure 6.** Targeted RNA degradation by bifunctional RNA-binding small molecules. (**A**) Following a mechanism similar to that of PROTACs to induce the target protein degradation, a RiboTAC molecule binds with the target RNA motif and recruits the RNase L, which dimerizes and cleaves the RNA. The chemical structures of three representative RiboTACs are shown on the right. (**B**) Bleomycin-conjugated RNA-binding small molecules (RiboSNAP) can induce RNA cleavage. Bleomycin is activated by $Fe^{2+}$ and $O_2$, initiates a superoxide radical transfer, and finally induces the RNA strand breaks. (**C**) An imidazole conjugate used to induce the cleavage of target RNA. Imidazole acts as an RNase mimic and cleaves the RNA strand by a proton transfer mechanism, which imitates the catalytic site of RNase A. The filled pink circles represent the RNA-binding small molecules which are conjugated with other functional moieties.

technology called proteolysis targeting chimeras (PROTACs), with one modality (ARV-471) in a Phase 3 clinical trial and receiving FDA fast track designation for the treatment of ER +/HER2− metastatic breast cancer.[98] PROTACs are hetero-bifunctional molecules combining one ligand that binds to a protein of interest (POI) and another that recruits an E3 ubiquitin ligase. The PROTAC molecule acts as a glue to bring the POI and E3 into proximity, thus promoting the POI ubiquitination and inducing subsequent degradation by the proteasomes (Figure 6A). It is worth noting that PROTACs can be recycled after dissociation from the target, and each molecule catalyzes the degradation over several rounds. Inspired by PROTACs, various strategies have been applied to modulate the degradation of RNA targets by bifunctional small molecules. Three of these are discussed below.

**4.1. RiboTAC.** RiboTACs (ribonuclease targeting chimeras) are a class of RNA-binding small molecules with the addition of an RNA degradation modality.[99] RiboTACs are reminiscent of PROTACs, but they recruits endogenous RNase to degrade an RNA of interest (Figure 6A).[99] The Disney group initially reported the concept by coupling an RNA-binding molecule, Targaprimir-96, to a short oligonucleotide, 2′-5′$A_4$.[100] Targaprimir-96 was identified to target the Drosha processing site of microRNA pri-miR-96,[101] while 2′-5′$A_4$ is structurally similar to the oligoadenylate, an endogenous activator of RNase L.[102] Upon recognition of pri-miR-96 by a RiboTAC, the 2′-5′$A_4$ module recruits RNase L and activates it by dimerization (Figure


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6A). The initial demonstration of the RiboTAC resulted in selective cleavage of the miR-96 precursor in cancer cells in a catalytic and substoichiometric fashion. However, the poor membrane permeability of oligonucleotides limits RiboTAC's potential in therapeutic applications. In order to develop a RiboTAC with better drug-likeness, the small-molecule RNase L recruiter was employed.[103] RiboTACs with new recruiters were developed to target pre-miR-21, pre-miR-210, r(G4C2) repeat expansion, *SNCA* mRNA, and the SARS-CoV-2 RNA genome.[103−107]

Notably, RiboTACs generally possess higher potency and longer effects than the RNA-binding small molecules from which they are derived.[108] Take the Dovitinib RiboTAC for example: RiboTAC enhances the miRNA inhibitory activity by 25-fold and increases the selectivity to pre-miR-21 by 2500-fold in comparison to Dovitinib.[109] In vivo, the Dovitinib RiboTAC alleviates disease progression in mouse models of triple-negative breast cancer and Alport syndrome, both of which are caused by miR-21 overexpression.[109] On-demand degradation of RNA targets could also be achieved by inducible RiboTACs (iRiboTACs), in which a caged ribonuclease recruiter is decaged by a tumor-specific enzyme or metabolite such as NQO1 or $H_2O_2$.[110] Using a DNA-encoded library (DEL), Meyer et al. focused on identifying new RNase L binders to design the next-generation RiboTACs.[111] A candidate was validated to be able to activate RNase L, but the recruiter did not show promising activities against the miR-21-driven pathologies when incorporated into Dovitinib RiboTACs. Nevertheless, this study demonstrated that DEL technology might be utilized to screen small-molecule recruiters of ribonucleases, which is important to broaden the application of RiboTACs.

**4.2. Targeted RNA Cleavage by Bleomycin Conjugates (RiboSNAP).** Bleomycin is a clinically used antitumor drug that can catalyze the cleavage of both DNA and RNA. It was thought that the cleavage of RNA by bleomycin was more selective than that of DNA. Bleomycin requires both Fe(II) and $O_2$ as cofactors to generate an "activated" bleomycin. This activated bleomycin accepts an electron and a proton to form the intermediate bleomycin-Fe(II)-OOH, which is quite active and unstable, with a half-life of approximately 2 min at 4 °C. The active intermediate transfers the radical ·OOH to the ribose moiety of RNA nearby and forms a C4′ radical intermediate that can proceed with a strand scission reaction (Figure 6B). The SAR study revealed that the bithiazole tail, together with its positively charged groups, is responsible for DNA binding, and this module also defines the sequence specificity of RNA cleavage. It is worth noting that the affinity of bleomycin for RNA is very weak and relatively nonspecific. Li et al. coupled the RNA-binding small molecule Targaprimir-96 to the bithiazole tail and generated a bleomycin conjugate which can bind >100-fold more strongly to pri-miR-96.[80] The conjugate selectively cleaves pri-miR-96 in triple-negative breast cancer (TNBC) cells.[112] Following a similar strategy (coined RiboSNAP to differentiate from RiboTAC), Angelbello et al. made a bleomycin conjugate targeting the r(CUG) repeat expansion to treat myotonic dystrophy type 1 (DM1).[113] This molecular design, annotated as Cugamycin, was later deglycosylated by the inventor to further promote the cleavage selectivity to RNA over DNA.[114] Interesting, RNA cleavage by RiboSNAP occurred throughout the cells (e.g., cytosol, nucleus, organelles, etc.). In contrast, a RiboTAC downregulates only the target RNA in the cytosol where the RNase L is located.

**4.3. Imidazole-Induced RNA Degradation.** Imidazole is another small-molecule-based ribonuclease mimic that can couple with RNA-binding ligands for targeted RNA degradation. It is well known that RNase A contains two essential histidine residues in the active center to catalyze the cleavage of RNA. Giege et al. reported that the chemical compound imidazole, as the residue group of histidine, could imitate the ribonuclease activity and act as an artificial ribonuclease to cleave tRNA at the single-stranded region.[115] The mechanism was proposed as follows: an acidic imidazolium coordinates the RNA phosphorate group during the transition state, then induces the cleavage of the phosphodiester bond and proton transfer from 2′-OH of ribose to another basic imidazole (Figure 6C).[116] Although the free imidazole hydrolyzes RNA at concentrations above 100 mM, the hydrolysis activity can be dramatically enhanced by the conjugation of imidazole with spermine or polyamine.[116,117] It is speculated that the polycationic moiety interacts with the negatively charged RNA backbone and brings the conjugated imidazole residue into a close contact with the phosphodiester, thus promoting the RNA cleavage. Using tRNA as substrate, the cationic imidazoles achieved complete RNA cleavage at concentrations as low as 2.5 mM. Nevertheless, the cleavage promoted by the cationic moiety is nonselective, thus limiting its biomedical applications. The target specificity can be solved by coupling imidazole to an antisense oligonucleotide (ASO), thereby promoting a cleavage reaction on the targeting site of the ASO.[118−121] Due to poor drug-likeness properties, the oligonucleotide-based RNA binders were later replaced by RNA-binding small molecules like neomycin and phenazine analogs, which demonstrated the ability to induce the site-specific cleavage of HIV-1 TAR RNA in physiological conditions.[115,122] Mikutis et al. used the strategy to design two RNA degraders which target two different RNA structures: G-quadruplexes and the betacoronaviral pseudoknot (MTDB).[123] Both of them have been demonstrated to degrade their target precisely in the cellular models and in vivo SARS-CoV-2 infection models. Even though the imidazole-based RNA degradation technology remains premature, the obtained results are particularly encouraging for the design of small-molecule degraders considering the much smaller size of imidazole than bleomycin and RNase L recruiters.

## 5. RNA-BINDING SMALL MOLECULES IN DRUG DELIVERY

As described above, RNA has been emerging as a target of small molecules for the prevention and treatment of human disease; meanwhile, some types of RNA, such as siRNA, mRNA, and synthetic RNA circuits, are important therapeutic agents, too. However, due to the inherent features, including instability, negative charge, and large size of RNA, delivery is the major barrier that impedes the extensive application of RNA drugs. By interaction with RNA, small molecules might provide a means to manipulate the non-drug-like features of RNA and improve their delivery efficiency.

**5.1. RNA-Binding Small Molecules as Carriers for siRNA Delivery.** siRNA is a short double-stranded RNA molecule with a length of 19−25 bp. As a large (∼14 kDa), highly hydrophilic, and polyanionic macromolecule, siRNA is membrane impermeable and possesses poor pharmaceutical properties, thereby dramatically limiting its biomedical applications. Significant efforts have been directed toward increasing the hydrophobicity of siRNA, with the aim of improving the membrane permeability and delivery efficiency.


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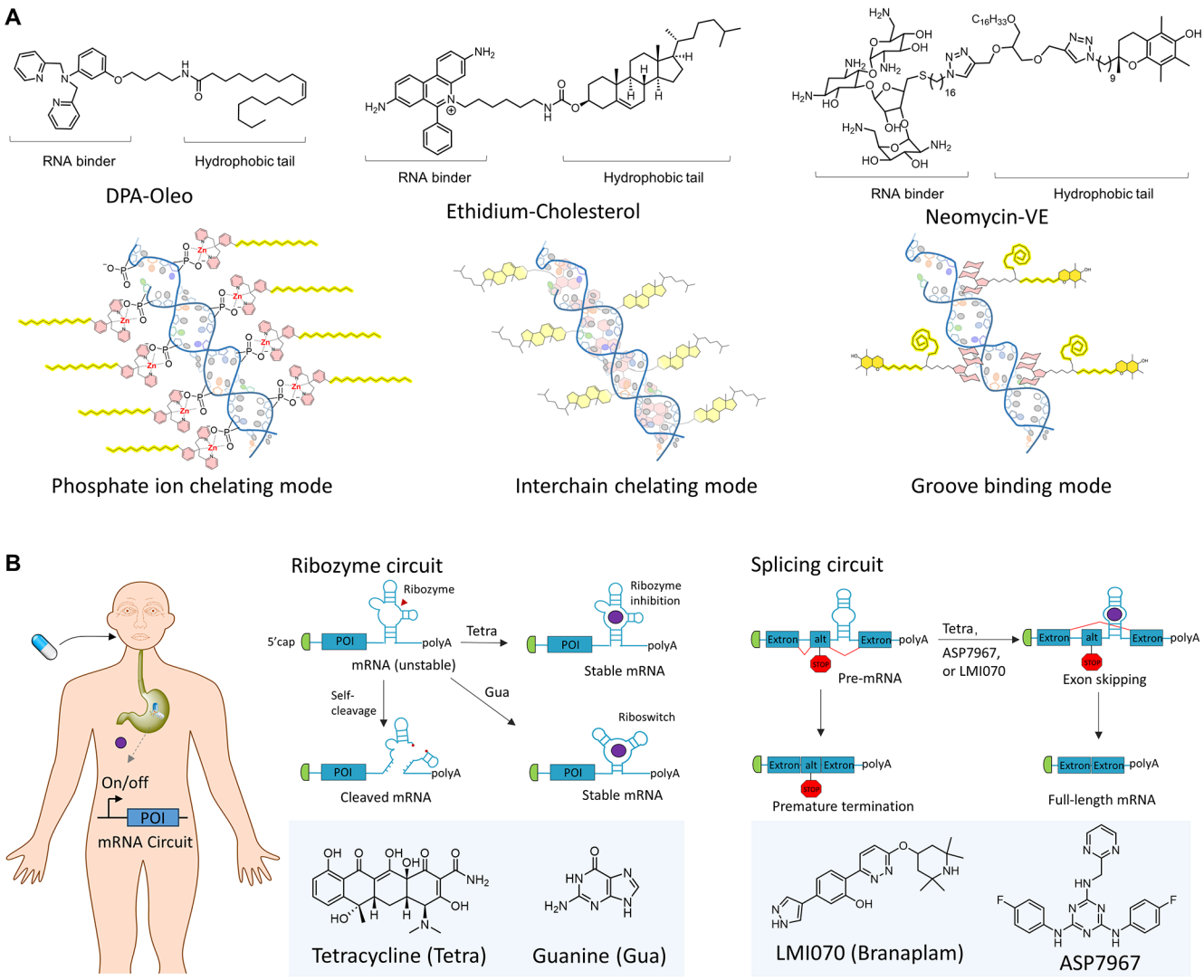


**Figure 7.** RNA-binding small molecules meditate the delivery of drugs. (**A**) RNA-binding small molecules conjugate with lipidoids and decorate the siRNA backbone to modulate the siRNA hydrophobicity, which can promote the self-delivery of siRNA to cells. (**B**) Oral administration of RNA-binding small molecules enables timely regulation of the in situ synthesis and delivery of drugs (therapeutic proteins) by the mRNA circuit. The genetic circuits are designed either by ribozymes to control mRNA stability or by RNA aptamers to regulate the splicing pattern of pre-mRNA. The small-molecule modulators of mRNA circuits are shown at the bottom in gray boxes.

Surface decoration by RNA-binding small molecules is one of these strategies that has received growing attention (Figure 7A). Kim et al. developed a dipicolylamine (DPA)-Oleo molecule that can bind to the phosphate backbones of siRNA with the coordination of zinc ions ($Zn^{2+}$).[124] Once chelated on the siRNA surface, the tagging molecule DAP-Oleo not only protects siRNA from degradation by RNases but also makes siRNA partially hydrophobic and membrane permeable, which achieves a moderate transfection efficiency. With concerns about the heavy-metal-induced toxicity, our lab has synthesized another siRNA-binding small molecule, ethidium−cholesterol, to manipulate siRNA's hydrophobicity.[125] This molecule utilizes the ethidium−siRNA duplex intercalation to decorate the hydrophobic moiety cholesterol on the siRNA surface, thus making the tagged siRNA membrane permeable and self-deliverable to cells.[126,127] As a cholesterol tagging technology alternative to the well-known cholesterol−siRNA conjugates, the noncovalent decoration of siRNA by this molecule is much easier to prepare, diversifiable in structure, and tunable in cholesterol/siRNA ratio, which encourages us to apply it for local delivery of siRNA to treat retinal diseases (unpublished).

Based on the same concept, the Wada group and the Pitard group independently developed another type of tagging molecule that utilizes neomycin as the siRNA-binding module.[128,129] Neomycin is one type of aminoglycoside that has been reported to bind with a variety of RNA secondary structures, including RNA duplexes.[130−132] With siRNA, neomycin stacks in the A-form major groove.[29,133] Due to the limited number of groove binding sites in the siRNA backbone, the authors introduced an $\alpha$-tocopherol (vitamin E, VE) and a long-chain alkyl group to the neomycin scaffold simultaneously to maximize the decoration effect. The neomycin-tagged siRNA demonstrated significant RNAi activity in liver cancer cells without using transfection reagents. It is worth noting that all the RNA-binding small molecules in Figure 6A are not binding-site specific, which may cause an off-target concern when they are applied in vivo.[134] An effort to screen small molecules that bind


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more specifically to siRNA is prioritized to promote this technology.

**5.2. RNA-Binding Small Molecules Regulating In Situ Drug Synthesis and Delivery.** Gene therapy holds great potential for future medical applications. In order to tightly control gene therapy, it is encouraging that patients would be able to switch-on the expression of mRNA-encoded therapeutics on demand by the temporal intake of a small-molecule drug.[135] RNA-binding small molecules can be employed as trigger signals to regulate the engineered mRNA circuits to obtain the desired outputs in a controlled and predictable manner (Figure 7B). Insertion of ribozymes or RNA aptamers as regulators in the 5′ or 3′ UTR of mRNA is a well-established strategy to regulate gene expression by a small molecule. A ribozyme is a small catalytic RNA motif that promotes the self-cleavage of its own sequence at a specific site. Once incorporated into the mRNA circuit, a ribozyme may influence the mRNA fate in two ways. Without small-molecule binding, the ribozyme would elicit its self-cleavage activity and shorten the half-life of mRNA, resulting in the switch-off of POI expression (Figure 6B). However, the mRNA's fate can be changed by applying small molecules to interrupt the ribozyme's activity, such as tetracycline inhibiting ribozyme K19's enzymatic activity[136] or guanine (Gua) triggering a riboswitch to disrupt the ribozyme fold.[137] These RNA-binding small molecules suppress the ribozyme activity, preventing mRNA from cleavage, therefore extending the half-life and allowing the translation of POI. Using these small-molecule regulators, an ON/OFF ratio up to 15 was achieved reversibly and repeatedly in mammalian cells.[136]

Regulation of the mRNA splicing pattern by RNA-binding small molecules is another type of mRNA circuit to regulate in situ drug synthesis and delivery. This concept was inspired by the fact that eukaryotic riboswitches regulate mRNA translation by controlling alternative splicing: homologs of bacterial TPP riboswitches control translation initiation (shown in Figure 3B).[52] By exploiting this method in the mRNA circuit, an alternative exon (alt) carrying a stop codon (red hexagon) was inserted before a riboswitch aptamer. In the presence of small-molecule ligands (such as tetracycline or LMI070), exon skipping is triggered, leading to the exclusion of the alternative exon and the expression of the functional full-length protein. Otherwise, constitutive splicing incorporates the alternative exon into the mRNA, which stops the protein translation prematurely. The dose or concentration required for optimal circuit regulation is highly dependent on the small-molecule ligand/aptamer pairs. For example, tetracycline induced an effective exon skipping at concentrations ranging from 5 to 100 $\mu$M,[138] while LMI070 (Branaplam) was effective at a concentration as low as 25 nM.[139] The robust control of gene expression by LMI070 enables a repeated regulation of protein expression in mice by oral administration, which paved the road for future preclinical studies.[139]

It is noted that a tighter regulation of gene expression can be obtained by combining ribozyme-based and exon-skipping riboswitch collectively. With just one mammalian riboswitch alone, either a ribozyme circuit or a splicing circuit, only a moderate ON/OFF ratio of 10−20 can be achieved. However, an ON/OFF ratio approaching 300 was achieved in the Yokobayashi lab by using the small-molecule inducer ASP7967 to regulate ribozyme and splicing circuits simultaneously in HEK293 cells.[140]

## 6. CONCLUSION

Cellular RNA is generally encoded by gigabase-sized genomic sequences, which can yield millions of RNA molecules. For each RNA molecule, the structure is far from static. Multiple alternative structures can coexist as heterogeneous and dynamic ensembles. Their flexible structures have long been known to be deeply intertwined with RNA functions.[141] Variations in sequence, structure, and function have RNA complexity at their core. As a less-explored field, RNA provides opportunities to expand the plethora of classical therapeutic targets but also poses challenges to develop small-molecule drugs targeting it. Nevertheless, classic phenotypic screening and emerging new technologies have dramatically accelerated the progress to identify lead compounds that can bind to various RNA motifs. Meanwhile, RNA-binding small molecules have also been exploited as tools to deliver and regulate RNA-based therapeutics. Their potential in gene therapy should never be disregarded.

The drug-likeness of lead compounds is also important when developing RNA-binding small molecules for therapy. With insights into the molecules obtained from DEL, Chem-CLIP, and Inforna screening, the chemical properties reveal some degree of structural similarity.[24,142] For example, scaffolds like phenylbenzimidazoles, 2-aminoquinazolines, 4,6-diaminopyrimidines, and 2-guanidinothiazoles were often seen in the structures of lead candidates when the screening was applied to the precursor microRNA-96, the microRNA-377 precursor, and other RNA motifs.[108,142,143] These features correlate to the bias of RNA-binding small molecules to interact with RNA via $\pi$-stacking and hydrogen bonds. However, this propensity may lead to the molecules being structurally planar and rigid, which might impair their oral bioavailability and drug-likeness. This problem might be solved with the expansion of the library size and chemotype in the future. Another challenge will be the issues of selectivity and specificity during small molecule/RNA binding. High-throughput screening methods, including Chem-CLIP, are effective at identifying new chemotypes to bind with secondary RNA structures. Considering the gigabase-sized sequences and countless structures of RNA in cells, it is worrying that the small-molecule leads likely bind more than one site and recognize multiple RNA targets simultaneously, which eventually may cause off-target toxicity. Further studies, therefore, are needed, especially chemical optimization, target validation, and structural biology, to determine the biological specificity and in vivo activity.

Compared with the prosperity of protein-targeting chemical drugs, the field of RNA-binding small molecules is still in its infancy. The FDA has already approved aminoglycosides as antibiotics and Risdiplam for SMA treatment. Some other candidates, such as Zotatifin, are undergoing clinical trials. As our understanding of RNA biology advances, the future is exciting for the field of RNA-binding small molecules.

## AUTHOR INFORMATION

### Corresponding Authors

**Wanyi Tai** − *Department of Pharmaceutical Engineering, School of Pharmaceutical Sciences, Wuhan University, Wuhan, Hubei 430071, China; Department of Pharmacy, Zhongnan Hospital of Wuhan University, Wuhan, Hubei 430071, China;* orcid.org/0000-0003-3589-8263; Email: wanyi-tai@whu.edu.cn



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**Hong Cheng** − *Department of Pharmaceutical Engineering, School of Pharmaceutical Sciences, Wuhan University, Wuhan, Hubei 430071, China; Department of Pharmacy, Zhongnan Hospital of Wuhan University, Wuhan, Hubei 430071, China*; Email: chenghong@znhostital.cn

### Authors

**Siyi Chen** − *Department of Pharmaceutical Engineering, School of Pharmaceutical Sciences, Wuhan University, Wuhan, Hubei 430071, China; Department of Pharmacy, Zhongnan Hospital of Wuhan University, Wuhan, Hubei 430071, China*

**Qi Mao** − *Department of Pharmaceutical Engineering, School of Pharmaceutical Sciences, Wuhan University, Wuhan, Hubei 430071, China; Department of Pharmacy, Zhongnan Hospital of Wuhan University, Wuhan, Hubei 430071, China*

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.jmedchem.4c01330

### Notes

The authors declare no competing financial interest.

### Biographies

**Siyi Chen** received her Bachelor of Science degree in Chemistry from Wofford College in 2015. She subsequently earned her Pharm. D. degree from Creighton University in 2019. She is currently a postdoctoral fellow in the Zhongnan Hospital of Wuhan University. Her research mainly focuses on the development of RNA-binding small molecules for siRNA delivery.

**Qi Mao** received his M.S. degree from Soochow University. He is currently a Ph.D. candidate at Wuhan University. His current focus is on the design of small-molecule tags for siRNA delivery.

**Hong Cheng** received her Master's degree in Pharmacology and her M.D. from Wuhan University. She is currently a researcher and pharmacist at Zhongnan Hospital of Wuhan University, where she focuses on the pharmacological mechanisms of novel bioactive molecules and conducts real-world studies on drug efficacy and safety.

**Wanyi Tai** earned his Ph.D. in Pharmaceutical Sciences from the University of Missouri−Kansas City. After graduation, he got his postdoctoral training at UNC-Chapel Hill and the University of Washington separately. He currently is a professor and doctoral tutor in the School of Pharmaceutical Sciences, Wuhan University, mainly focusing on drug bioconjugation and delivery.

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## ■ ABBREVIATIONS USED

snoRNA, small nucleolar RNA; snRNA, small nuclear RNA; lncRNA, long noncoding RNA; ASO, antisense oligonucleotide; rRNA, ribosomal RNA; SMA, spinal muscular atrophy; FMN, flavin mononucleotide; IRES, internal ribosome entry site; pri-miRNA, primary miRNA; pre-miRNA, precursor miRNA; UTR, untranslated region; RBS, ribosomal binding site; SRE, splicing regulatory element; SAR, structure−activity relationship; SMN, survival motor neuron; NMR, nuclear magnetic resonance; RTK, receptor tyrosine kinase; CDK, cyclin-dependent kinase; eIF4A, eukaryotic translation initiation factor 4A; eRF1, eukaryotic release factor; PROTAC, proteolysis targeting chimera; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; POI, protein of interest; RiboTAC, ribonuclease targeting chimera; DEL, DNA-encoded library; TNBC, triple-negative breast cancer; DM1, myotonic dystrophy type 1; DPA, dipicolylamine; Gua, guanine; Chem-CLIP, chemical cross-linking and isolation by pull-down

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