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RNA Drugs and RNA Targets for Small Molecules: Principles, Progress, and Challenges

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Abstract——RNA-based therapies, including RNA molecules as drugs and RNA-targeted small molecules, offer unique opportunities to expand the range of therapeutic targets. Various forms of RNAs may be used to selectively act on proteins, transcripts, and genes that cannot be targeted by conventional small molecules or proteins. Although development of RNA drugs faces unparalleled challenges, many strategies have been developed to improve RNA metabolic stability and intracellular delivery. A number of RNA drugs have been approved for medical use, including aptamers (e.g., pegaptanib) that mechanistically act on protein target and small interfering RNAs (e.g., patisiran and givosiran) and antisense oligonucleotides (e.g., inotersen and golodirsen) that directly interfere with RNA targets. Furthermore, guide RNAs are essential components of novel gene editing modalities, and mRNA therapeutics are under development for protein replacement therapy or vaccination, including those against unprecedented severe acute respiratory syndrome coronavirus pandemic. Moreover, functional RNAs or RNA motifs are highly structured to form binding pockets or clefts that are accessible by small molecules. Many natural, semisynthetic, or synthetic antibiotics (e.g., aminoglycosides, tetracyclines, macrolides, oxazolidinones, and phenicols) can directly bind to ribosomal RNAs to achieve the inhibition of bacterial infections. Therefore, there is growing interest in developing RNA-targeted small-molecule drugs amenable to oral administration, and some (e.g., risdiplam and branaplam) have entered clinical trials. Here, we review the pharmacology of novel RNA drugs and RNA-targeted small-molecule medications, with a focus on recent progresses and strategies. Challenges in the development of novel druggable RNA entities and identification of viable RNA targets and selective small-molecule binders are discussed.

Significance Statement——With the understanding of RNA functions and critical roles in diseases, as well as the development of RNA-related technologies, there is growing interest in developing novel RNA-based therapeutics. This comprehensive review presents pharmacology of both RNA drugs and RNA-targeted small-molecule medications, focusing on novel mechanisms of action, the most recent progress, and existing challenges.

I. Introduction

Therapeutic drugs act on corresponding molecular targets, biological pathways, or cellular processes to elicit pharmacological effects for the treatment of human diseases. Small-molecule compounds and proteins/antibodies remain as the major forms of medications for medical use and the preferred modalities in drug development, acting mainly on protein targets such as enzymes, receptors, ion channels, transporters, and kinases (Santos et al., 2017; Usmani et al., 2017; Rock and Foti, 2019; Yin and Rogge, 2019). With unique physicochemical and pharmacological characteristics complementary to traditional protein-targeted small-molecule and protein drugs (Table 1), RNA molecules, such as aptamers, antisense oligonucleotides (ASO), small interfering RNAs (siRNA), and guide RNAs (gRNA), have emerged as a new class of modalities in clinical practice and are under active development (Crooke et al., 2018; Yin and Rogge, 2019; Yu et al., 2019); RNA molecules may act not only on conventional proteome but also on previously undrugged transcriptome, including mRNAs to be translated into proteins and functional noncoding RNAs (ncRNAs) which largely outnumber mRNAs (Mattick, 2004; Djebali et al., 2012), as well as the genome. Moreover, some mRNAs and ncRNAs, such as microRNAs (miRNA or miR), are also under preclinical and clinical development for replacement therapy or vaccination

TABLE 1
Characteristics of inorganic and small-molecule organic compound drugs, as well as macromolecule protein and nucleic acid therapeutics

Properties	Inorganic Compound Drugs	Small-Molecule Organic Compound Drugs	Protein Therapeutics	RNA Therapeutics
Chemistry	Typical mol. wt. < 200 Da; ionic	Typical mol. wt. < 500 Da; hydrophobic	Typical mol. wt. > 100 kDa; positive/negative/neutral	Typical mol. wt. > 7 kDa; negative charge
Dosing	Primarily oral; often daily	Primarily oral; often daily	Mainly intravenous and subcutaneous; weekly to monthly	Intravenous, subcutaneous, intrathecal, intravitreal (various); weekly to once every 3–6 mo
ADME/PK properties	Orally bioavailable; distributed to all organs and tissues, cell permeable; usually not metabolized; excreted primarily in urine	Orally bioavailable; distributed to all organs and tissues, cell permeable; metabolized by phase I and II enzymes; excreted mainly in bile and urine	Not orally bioavailable; distributed mainly in plasma or extracellular fluids, cell impermeable; catabolized extensively to peptides or amino acids; limited excretion	Not orally bioavailable; Distributed extensively to kidney and liver, cell impermeable; catabolized extensively by nucleases to (oligo)nucleotides; limited excretion
Molecular targets	Proteins	Mainly proteins	Proteins	Mainly RNAs, besides proteins and DNAs
Site of action and PD	Extra-/intracellular; direct or indirect relationship to blood PK	Extra-/intracellular; Direct or indirect relationship to blood PK	Extracellular/membrane; direct or indirect models linked to blood PK	Primarily intracellular; more relevant to tissue PK, whereas PD can be linked to blood PK
Safety/ toxicity	Risk of off-target effects	Risk of off-target effects	Risk of immunogenicity	Risk of immunogenicity

ADME, absorption, metabolism, distribution, and excretion.

(Bader et al., 2010; Sahin et al., 2014; Lieberman, 2018), and new approaches and technologies are emerging to tackle some inherited or overlooked issues, such as the choice of RNA molecules (Ho and Yu, 2016; Yu et al., 2019). On the other hand, traditional small-molecule compounds may be employed to directly target pathogenic RNAs for the treatment of diseases (Donlic and Hargrove, 2018; Warner et al., 2018; Costales et al., 2020), providing another unparalleled opportunity to expand the range of therapeutic targets.

In this review, we first provide a classification of RNA-based therapeutics, RNA drugs, and RNA-targeted small molecules and describe their general characteristics. After summarizing the promise of RNA drugs, we review specific types of RNA drugs and their novel mechanisms of action and discuss major barriers in the development of RNA therapeutics, as well as respective proven and potential strategies. Next, we summarize the strategies and potential in developing RNA-targeted small-molecule drugs, including recent advances in structural understanding of antibiotic–ribosomal RNA (rRNA) interactions and identification of novel RNA-binding small molecules. Challenges in the identification of therapeutic RNA targets and determination of the selectivity of RNA–small-molecule interactions for the control of specific diseases are also discussed.

II. Classification and General Features of RNA-Based Therapeutics

RNA-based therapeutics are classified as two types of entities: RNA molecules or analogs directly used as therapeutic drugs (Kole et al., 2012; Sahin et al., 2014; Crooke et al., 2018; Yu et al., 2019) and RNA-targeted small-molecule medications (Donlic and Hargrove, 2018; Warner et al., 2018; Costales et al., 2020). Firstly, a number

of RNA drugs have been approved by the US Food and Drug Administration (FDA) for the treatment of various human diseases, including RNA aptamers (e.g., pegaptanib) (Gragoudas et al., 2004; Gryziewicz, 2005), ASOs or antisense RNAs (asRNAs) (e.g., mipomersen, eteplirsen, nusinersen, inotersen, and golodirsen) (Morrow, 2013; Stein, 2016; Syed, 2016; Ottesen, 2017; Keam, 2018), and siRNAs (e.g., patisiran and givosiran) (Wood, 2018; Scott, 2020) (Table 2; this review). RNA molecules have appeared to be highly specific in acting on a wide variety of proven and possible therapeutic targets, including proteins, transcripts, and genes (Table 1), that may not be accessible by small-molecule compounds and proteins. Nevertheless, RNAs are prone to catabolism by serum RNases and are required to pass the cellular membrane barriers to access intracellular targets. Similar to protein therapeutics (e.g., insulin, trastuzumab, and pembrolizumab, etc.), RNA drugs (e.g., mipomersen and patisiran, etc.) are not orally bioavailable; hence, both RNA and protein drugs are usually administered to patients via other routes, such as intravenous or subcutaneous injection (Table 1). This is totally different from many small-molecule inorganic (e.g., lithium carbonate) and organic (e.g., acetaminophen, dextromethorphan, and ibuprofen, etc.) compound drugs, which exhibit favorable or acceptable oral bioavailability and are primarily administered orally to patients.

Secondly, conventional small-molecule compounds with broad structural diversities and drug-like physicochemical and PK properties are preferred entities to bind and manipulate highly structured RNA targets (Hermann, 2016; Donlic and Hargrove, 2018; Warner et al., 2018; Costales et al., 2020). After the identification of an RNA target, selection of proper drug-like small molecules, and determination of RNA–small-molecule interactions (e.g., binding affinity and selectivity), the

TABLE 2
RNA therapeutics approved by the US Food and Drug Administration for the treatment of human diseases
Note that two PMO drugs are included because the nucleobase thymine (T) is also known as 5-methyluracil, or m^5U.

RNA Drug	Chemistry	Dosage Regimen	Mechanisms of Action	Disease	Year Approved	Current Status	References
Pegaptanib (aptamer)	28-nt aptamer; pegylated, all PO, 2′-F, and 2′-OMe; G and A methylated; mol. wt. ~50 kDa	0.3 mg every 6 wk, intravitreal injection	Selective VEGF (165 isoform) antagonist; antiangiogenesis in the eye	Neovascular AMD	2004	Prescription	Gragoudas et al., 2004; Gryziewicz, 2005
Mipomersen (ASO)	20-mer gapmer; all PS, 2′-MOE, and 2′-deoxy; C and U methylated; mol. wt. ~7.6 kDa	200 mg once weekly, s.c.	Selectively binds to ApoB-100 mRNA to inhibit the translation of synthesis of ApoB in liver	HoFH	2013	Discontinued in 2018	Crooke and Geary, 2013; Morrow, 2013
Eteplirsen (ASO)	30-mer PMO; m^5U; mol. wt. ~10.3 kDa	30 mg/kg once weekly, i.v. infusion	Selectively binds to exon 51 of dystrophin pre-mRNA to alter splicing, leading to production of functional muscle protein dystrophin	DMD	2016	Prescription	Cirak et al., 2011; Mendell et al., 2016; Stein, 2016; Syed, 2016
Nusinersen (ASO)	18-mer; all PS, fully 2′-MOE; m^5U; m^5C; mol. wt. ~7.5 kDa	Loading: 12 mg every 2 wk for three doses, then 12 mg for 30 days, i.t. Maintenance: 12 mg once every 4 mo, i.t.	Selectively binds to SMN2 mRNA to alter splicing, leading to the production of full-length SMN protein	SMA	2016	Prescription	Aartsma-Rus, 2017; Ottesen, 2017
Patisiran (siRNA)	21-bp double-stranded siRNA; all PO, and 2′-OMe; lipid nanoparticle mol. wt. ~14.3 kDa	0.3 mg/kg (b.wt. < 100 kg) or 30 mg (b.wt. ≥ 100 kg), every 3 wk, i.v. infusion	Selectively binds to TTR mRNA to decrease hepatic production of TTR protein	hATTR amyloidosis	2018	Prescription	Adams et al., 2018; Wood, 2018; Zhang et al., 2020b
Inotersen (ASO)	20-mer gapmer; all PS, 2′-MOE, and 2′-deoxy; C and U methylated; mol. wt. ~7.2 kDa	284 mg once weekly, s.c.	Selectively binds to TTR mRNA to cause mRNA degradation and reduce protein production	hATTR amyloidosis	2018	Prescription	Benson et al., 2018; Keam, 2018
Givosiran (siRNA)	Double-stranded siRNA; PO and PS, 2-F′, 2′-O-Me, and triantennary GalNAc; mol. wt. ~16.3 kDa	2.5 mg/kg once monthly, s.c.	Selectively binds to hepatic ALAS1 mRNA, leading to ALAS1 mRNA degradation through RNA interference	AHP	2019	Prescription	Sardh et al., 2019; de Paula Brandao et al., 2020; Scott, 2020
Golodirsen	25-mer PMO; m^5U; mol. wt. ~8.6 kDa	30 mg/kg once weekly, i.v. infusion	Selectively binds to exon 53 of dystrophin pre-mRNA to alter splicing, leading to production of functional muscle protein dystrophin in patients with genetic mutations that are amenable to exon 53 skipping	DMD	2019	Prescription	Heo, 2020

2′-F, 2′-fluoro; 2′-OMe, 2′-methoxy.

RNA-targeted small molecules may be processed for further preclinical and clinical investigations to define efficacy and safety profiles. Supporting this concept, many antibiotic drugs, such as natural and semisynthetic aminoglycosides (e.g., streptomycin, paromomycin, neomycin, etc.) (Fourmy et al., 1996; Ogle et al., 2001; Demeshkina et al., 2012; Demirci et al., 2013), tetracyclines (e.g., tetracycline, tigecycline, etc.) (Brodersen et al., 2000; Anokhina et al., 2004; Schedlbauer et al., 2015), and macrolides (erythromycin, azithromycin, telithromycin, etc.) (Vannuffel and Cocito, 1996; Hansen et al., 2002; Berisio et al., 2003; Tu et al., 2005; Bulkley et al., 2010), as well as synthetic oxazolidinones (e.g., linezolid, etc.) (Ippolito et al., 2008; Wilson et al., 2008), being approved for clinical use have been revealed to mechanistically bind to rRNAs within the 30S or 50S subunits to interfere with protein synthesis for the control of infections (Wilson, 2009, 2014; Lin et al., 2018). Therefore, large efforts are underway to identify viable RNA targets and assess new RNA-targeted small molecules for the treatment of various types of human diseases (Warner et al., 2018).

III. RNAs as Therapeutic Drugs

A. The Rise and Promise of RNA Therapeutics

With the understanding of new biological processes and development of novel technologies, such as those for gene silencing and genome editing (Stephenson and Zamecnik, 1978; Zamecnik and Stephenson, 1978;

Lee et al., 1993; Wightman et al., 1993; Fire et al., 1998; Jinek et al., 2012; Cong et al., 2013; Mali et al., 2013), diverse RNA molecules have been used to interfere with potential therapeutic targets (Fig. 1). Firstly, RNA aptamers can directly bind to extracellular, cell surface, or intracellular proteins (Gragoudas et al., 2004; Gryziewicz, 2005) that are traditionally targeted by small-molecule and protein drugs. Secondly, ASOs or asRNAs, siRNAs, and miRNA mimics may be delivered into cells to target intracellular mRNAs or functional ncRNAs through complementary base pairings, leading to gene silencing or control of gene expression for the treatment of diseases. Thirdly, a sense RNA or mRNA molecule can be introduced into cells and then translated into target proteins for protein replacement therapy or vaccination (Sahin et al., 2014; Lieberman, 2018). In addition, the genetic sequences dictating disease initiation and progression may be directly changed by using proper gRNAs and other necessary components to achieve eradication of the disease. As such, RNAs are unique molecules that are able to interact with three major forms of biological macromolecules—DNAs, RNAs, and proteins (Fig. 1)—and the development of RNA therapeutics is expected to expand the range of druggable targets, including conventional proteins and previously undrugged or "undruggable" transcripts and genes.

The development of novel RNA therapeutics has proven highly challenging given the fact that RNA drugs are anticipated to act primarily on intracellular targets (Fig. 1) and that RNA molecules exhibit "undrug-like" physicochemical and PK properties, especially when compared with small-molecule compounds (Table 1).
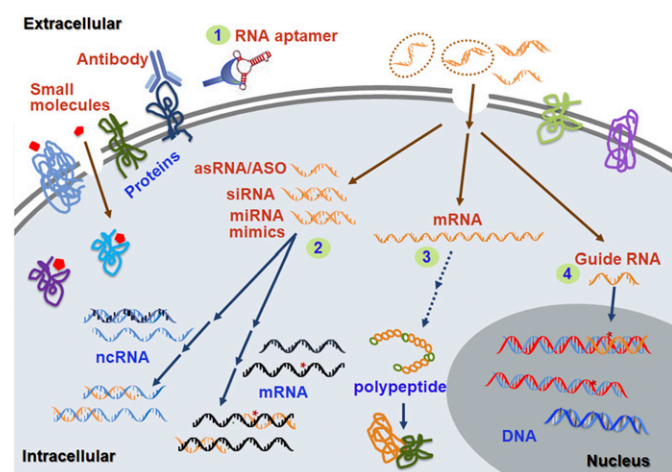

Fig. 1. RNA therapeutics are expected to expand the range of druggable targets from proteins to RNAs and DNAs. Cell surface, extracellular, and intracellular proteins remain favorable targets for the development of small-molecule and protein (e.g., antibody) therapeutics, as well as RNA aptamer drugs (1). Actually, the majority of human genome sequences transcribed as functional ncRNAs largely outnumbered mRNAs to be translated into proteins. Both mRNAs and ncRNAs can be directly targeted by RNA drugs such as ASO/asRNAs, miRNAs, and siRNAs (2). Once introduced into cells, mRNA therapeutics (3) may be developed for protein replacement therapy or vaccination. In addition, gRNAs (4) could be used along with other elements to directly edit the target gene sequences for the treatment of particular diseases.

As a polymeric molecule consisting of a sequence of variable numbers of four major forms of ribonucleotides that differ in their nucleobases, adenine (A), uracil (U), guanine (G), and cytosine (C), naked and unmodified RNAs are extremely susceptible to hydrolysis by non-specific RNases (e.g., RNase A) that are highly abundant in the blood (Houseley and Tollervey, 2009). Furthermore, RNAs are large molecules (e.g., with molecular weights >7 kDa) and are negatively charged (Table 1); thus, it is hard for them to cross the cell membrane. In addition, upon entering into cells, exogenous RNAs need to escape from endosomal trapping and/or degradation by large classes of intracellular ribonucleases or RNases (e.g., endonucleases, and 5′ and 3′ exonucleases), become incorporated into specific complex, and get access to the targets (Fig. 1) to exercise pharmacological effects.

With the development of new strategies to improve the druggability of RNA molecules, as well as the understanding of mechanisms of action, a number of RNA analog drugs have been approved by the FDA for the treatment of human diseases (Table 2), and many others are under active trials [for recent reviews, see Kowalski et al. (2019), Yu et al. (2019)]. The approval of the first RNA aptamer drug, pegaptanib (Gragoudas et al., 2004; Gryziewicz, 2005), supports the concept of using RNA molecules to inhibit protein targets (Fig. 1). Through specific chemical modifications to improve metabolic stability, targeting, binding affinity, and silencing efficacy (Eckstein, 1985; Campbell et al., 1990; Wheeler et al., 2012; Summerton, 2017; Crooke et al., 2018), ASOs [including "gapmers" and phosphorodiamidate morpholino oligomers (PMOs)] have become the most successful class of RNA drugs (e.g., mipomersen, eteplirsen, nusinersen, inotersen, and golodirsen) (Table 2). Furthermore, the most recent approval of two siRNA drugs, patisiran (Adams et al., 2018; Wood, 2018) and givosiran (Scott, 2020), not only testifies to the benefits of developing new approaches to improve the PK and pharmacodynamics (PD) properties of RNA drugs (Nair et al., 2017) but also supports the potential of RNA therapeutics.

B. Types of RNA Drugs and Mechanisms of Action

1. Antisense Oligonucleotides. The use of chemically synthesized, single-stranded oligonucleotide to selectively inhibit target gene expression via complementary base pairings with targeted mRNA was first reported in 1978 (Stephenson and Zamecnik, 1978; Zamecnik and Stephenson, 1978). Since then, ASOs have been widely used for the study of gene functions and development of novel therapeutics [for reviews, see Kole et al. (2012), Bennett (2019), Levin (2019)]. This is in parallel with the discovery of the presence of natural asRNAs in virtually all species (Spiegelman et al., 1972; Light and Molin, 1983; Simons and Kleckner, 1983; Izant and Weintraub, 1984; Ecker and Davis, 1986) and thus broad recognition of their critical functions in post-transcriptional gene regulation [for reviews,

see Vanhée-Brossollet and Vaquero (1998), Pelechano and Steinmetz (2013), Nishizawa et al. (2015)]. The knockdown of target gene expression with ASOs includes RNase-dependent cleavage of mRNAs and precursor mRNAs (pre-mRNAs) (RNase H and RNase P), as well as RNase-independent suppression of protein synthesis. Furthermore, ASOs can be employed to modulate RNA splicing to produce functional proteins or preferred genetic products (Condon and Bennett, 1996; McClorey et al., 2006). Although they differ from siRNAs and miRNAs that rely mainly on cytoplasmic siRNA-induced silencing complexes (RISCs)/miRNA-induced silencing complexes to control target gene expression, ASOs designed to target the same sites of common transcripts could be equally active as siRNAs, with some exceptions (Vickers et al., 2003). In addition, ASOs seem to be more effective to knock down nuclear targets, whereas siRNAs are superior at suppressing cytoplasmic targets (Lennox and Behlke, 2016), likely due to the fact that RNases H & P are highly abundant in the nucleus and RISCs are present within the cytoplasm.

To make ASOs druggable, a wide variety of chemical modifications have been developed to improve their metabolic stability and cell penetration efficiency, including the change of phosphodiester (PO) linker to phosphorothioate (PS), protection of the 2′-hydroxyl group on ribose with methyl (2′-methoxy) or methoxyethyl (2′-MOE), or direct substitution with fluorine (2′-fluoro), and connection of the 2′-O and 4′-C with a methylene bridge, namely locked nucleic acid [for reviews, see Ho and Yu (2016), Khvorova and Watts (2017), Crooke et al. (2018), Yu et al. (2019)]. More extensive modifications are also established for ASOs, among which the nucleobases are retained for base pairings while the ribose 5-phosphate linkages may be fully substituted with morpholino phosphorodiamidate backbones, leading to PMOs (Heasman et al., 2000). In addition, specific ligands, such as hepatocyte asialoglycoprotein receptor-binding N-acetylgalactosamine (GalNAc) (Nair et al., 2014, 2017), may be covalently attached to ASOs (and siRNAs, miRNAs, aptamers, etc.) to achieve cell- or organ-selective gene silencing. Some chemical modifications are proven to be very useful for the development of ASO drugs, as demonstrated by their utility in FDA-approved RNA drugs (Table 2).

Since the first ASO drug, fomivirsen, an antisense oligodeoxynucleotide (5′-GCG TTT GCT CTT CTT CTT GCG-3′) with phosphorothioate linkages, was approved by FDA in 1998 for the treatment of cytomegalovirus retinitis (Roehr, 1998), a number of ASO therapeutics have been successfully marketed in the United States (Table 2) (Mendell et al., 2013; Morrow, 2013; Robinson, 2013; Syed, 2016; Aartsma-Rus and Krieg, 2017; Ottesen, 2017; Stein and Castanotto, 2017; Benson et al., 2018; Keam, 2018; Wood, 2018). Among them, mipomersen (5′-G*-MeC*-MeC*-MeU*-MeC*-dA-dG-dT-dMeC-dG-dT-dMeC-dT-dT-dMeC-G*-MeC*-A*-MeC*-MeC*-3′; * = 2′-MOE; Me = 5-methyl, and d = deoxy; all PS linkages) and

inotersen (5′-MeU*-MeC*-MeU*-MeU*-G*-dG-dT-dT-dA-dMeC-dA-dT-dG-dA-dA-A*-MeU*-MeC*-MeC*-MeC*-3′; * = 2′-MOE; Me = 5-methyl, and d = deoxy; all PS linkages) are also known as gapmers, consisting of modified antisense oligoribonucleotides at both 5′ and 3′ ends with a "gap" of oligodeoxynucleotide in the middle. Upon selective binding to a targeted transcript, the resulting DNA-RNA heteroduplex is recognized by RNase H, leading to the cleavage of targeted RNA strand and the knockdown of targeted gene expression. Specifically, mipomersen has been shown to selectively bind to *ApoB-100* mRNA to reduce ApoB-100 protein levels, which is the major constituent of low-density lipoprotein (LDL); thus, it exhibits effectiveness for the treatment of patients with homozygous familial hypercholesterolemia (HoFH) (Stein et al., 2012; Crooke and Geary, 2013; Thomas et al., 2013). Rather, mipomersen was discontinued in 2018 because of competition from other therapeutics and an incapability of achieving marketing success (Yin and Rogge, 2019). On the other hand, inotersen selectively binds to transthyretin (TTR) mRNA to achieve the suppression of hepatic TTR protein expression levels and thus exerts therapeutic benefits among adults with hereditary transthyretin-mediated amyloidosis (hATTR amyloidosis) (Benson et al., 2018; Coelho et al., 2020).

Nusinersen (5′-UCA CUU UCA UAA UGC UGG-3′; fully modified with 2′-MOE and PS linkages; all Us and Cs are 5′-methylated) was approved by FDA in 2016 (Aartsma-Rus, 2017; Ottesen, 2017) for the treatment of a rare autosomal recessive neuromuscular disorder, spinal muscular atrophy (SMA), which is caused by genetic variations in the chromosome 5q11.2-q13.3 locus, affecting survival motor neuron (SMN) gene expression and leading to an insufficient level of SMN protein (Brzustowicz et al., 1990; Lefebvre et al., 1995). Nusinersen is an extensively modified 18-mer ASO whose PO linkages are completely changed to PS, and all ribose rings are protected with 2′-MOE (Table 2). Through the modulation of alternate splicing of SMN2 pre-mRNA to increase exon 7 inclusion to achieve the expression of full-length functional SMN protein (Rigo et al., 2014), nusinersen was found to be effective in improving patient survival or motor function (Finkel et al., 2017; Mercuri et al., 2018).

Eteplirsen and golodirsen are two PMO drugs (Table 2), approved in 2016 (Stein, 2016; Syed, 2016) and 2019 (Heo, 2020), respectively, for the treatment of Duchenne muscular dystrophy (DMD), a lethal neuromuscular disorder commonly caused by genetic mutations disrupting the reading frame of the X-linked dystrophin gene and which might be found in one of 3500 newborn boys (Cirak et al., 2011). Eteplirsen and golodirsen contain 30 and 25 linked PMO subunits whose sequences of bases are 5′-CUC CAA CAU CAA GGA AGA UGG CAU UUC UAG-3′ and 5′-GUU GCC UCC GGU UCU GAA GGU GUU C-3′, respectively, among which all Us are methylated or regarded as T. Both eteplirsen and golodirsen are designed

to restore the reading frame of dystrophin gene for the production of an internally truncated, yet functional, dystrophin protein. In particular, eteplirsen selectively binds to the exon 51 of dystrophin pre-mRNA (Popplewell et al., 2010), leading to exclusion of this exon during mRNA processing among patients with DMD showing genetic mutations amenable to exon 51 skipping (van Deutekom et al., 2007). With the production of functional dystrophin protein, eteplirsen-treated patients were shown to have a slower rate of decline in ambulation (Cirak et al., 2011; Mendell et al., 2013, 2016; Khan et al., 2019). Likewise, golodirsen selectively binds to the exon 53 of dystrophin pre-mRNA to achieve the exclusion of this exon during mRNA processing (Popplewell et al., 2010) and thus the expression of functional muscle protein dystrophin among patients with confirmed genetic mutations that are amenable to exon 53 skipping (Frank et al., 2020; Heo, 2020).

Many clinical trials are ongoing to investigate some new ASOs for the treatment of specific diseases, spanning from orphan genetic disorders to infectious diseases and cancers (https://www.clinicaltrials.gov/) (Yu et al., 2019). For instance, a deep intronic c.2991+1655A>G mutation in CEP290 underlying Leber congenital amaurosis type 10, an inherited retinal dystrophy, may be corrected by ASO therapy. Indeed, QR-110 was identified to effectively restore wild-type CEP290 mRNA and protein expression levels in CEP290 c.2991+1655A>G homozygous and heterozygous Leber congenital amaurosis type 10 primary fibroblasts as well as induced pluripotent stem cells–derived retinal organoids, and it was tolerated in monkeys after intravitreal injection (Dulla et al., 2018). Therefore, a double-masked, randomized, multiple-dose phase II/III study (NCT03913143) is underway to evaluate the efficacy, safety, tolerability, and systemic exposure of intravitreally administered QR-110 in patients with Leber congenital amaurosis who are amenable to the CEP290 p.Cys998X mutation. As another example, IONIS-AR-2.5Rx, a next-generation ASO against androgen receptor, has entered into a phase Ib/II single-arm study (NCT03300505) to identify an effective and safe dose level for the treatment of metastatic castration-resistant prostate cancer as combined with a fixed dose of enzalutamide.

2. Small Interfering RNAs. Since the discovery and development of RNA interference (RNAi) technologies with double-stranded RNAs (dsRNAs) (Fire et al., 1998; Zamore et al., 2000; Elbashir et al., 2001), 18- to 22-bp siRNAs have been routinely used for selective and effective knockdown of target gene expression in basic research, and some have entered clinical drug development [for reviews, see Castanotto and Rossi (2009), Setten et al. (2019), Yu et al. (2019)]. Different from the single-stranded ASO, siRNA comprises two strands, in which the guide strand is characterized by two 3′-overhang ribonucleotides crucial for the duration of gene silencing (Strapps et al., 2010). The endoribonuclease Dicer or helicase with RNase motif (Bernstein et al., 2001; Hutvágner et al., 2001) trims dsRNAs and separates the guide and passenger strands within the RISC (Hammond et al., 2000). The passenger strand is preferentially cleaved by the endonuclease argonaute-2 (Matranga et al., 2005; Rand et al., 2005), which is the catalytic core of RISC (Hammond et al., 2001; Martinez et al., 2002; Liu et al., 2004; Meister et al., 2004; Okamura et al., 2004; Rand et al., 2004). The guide strand containing a thermodynamically less stable 5′ end retained within the RISC (Khvorova et al., 2003; Schwarz et al., 2003) acts on its targeted mRNA through perfect complementary base pairings, leading to a sequence-specific cleavage of the targeted mRNA by argonaute-2 and consequently the knockdown of target gene. Because RISC is solely located within cytoplasm, whereas RNase H is predominately in nucleus, as mentioned previously, siRNAs are usually more effective than ASOs in knocking down cytoplasmic targets (Lennox and Behlke, 2016).

To develop siRNA therapeutics, it is essential to achieve potent, specific, and long-lasting gene silencing while minimizing off-target effects (Kim et al., 2005; Ui-Tei et al., 2008; Wang et al., 2009). With an improved understanding of RNAi mechanisms, some specific guidelines may be followed, and particular software can be used for the design of effective siRNAs [for reviews, see Jackson and Linsley (2010), Naito and Ui-Tei (2012), Fakhr et al. (2016)]. The selection of a proper target site, usually closer to the start codon within the coding sequence, is critical for the effectiveness of siRNA. This is also crucial to ensure selectivity and lessen off-target effects of siRNA. The composition of siRNA, such as the use of specific ribonucleotides at particular locations and overall G/C content, affects not only the stability but also the efficacy of siRNA. As siRNA may induce immune response in sequence-independent and sequence-dependent manners (Alexopoulou et al., 2001), it is important to avoid immune-stimulatory motifs such as U-rich sequences when designing siRNAs (Kleinman et al., 2008; Goodchild et al., 2009). In addition, similar to the development of ASO drugs, chemical modification is a common strategy to improve the metabolic stability and PK properties of siRNAs (Bramsen and Kjems, 2012; Yu et al., 2019). In any case, the effectiveness, selectivity, and safety of individual siRNAs require extensive and critical experimental validation.

The first siRNA drug, patisiran (Table 2), was approved by the FDA in 2018 for the treatment of the polyneuropathy of hATTR amyloidosis in adults (Wood, 2018; Yu et al., 2019) over 20 years after the discovery of siRNA-controlled gene silencing. hATTR amyloidosis is an autosomal-dominant, life-threatening disease caused by genetic mutations of *TTR*. As TTR protein is primarily produced in the liver, pathogenic mutations lead to misfolded TTR proteins that deposit as amyloid in peripheral nerves, heart, kidney, and gastrointestinal tract

(Adams et al., 2018; Zhang et al., 2020b). Patisiran is a 21-bp siRNA with extensive chemical modifications (Table 2) whose sense sequence is 5′-G-MeU-A-A-MeC-MeC-A-A-G-A-G-MeU-A-MeU-MeU-MeC-MeC-A-MeU-dT-dT-3′ and antisense sequence is 5′-A-U-G-G-A-A-MeU-A-C-U-C-U-U-G-G-U-MeU-A-C-dT-dT-3′ (Me = 2′-O-methyl, and d = deoxy), formulated as a lipid nanoparticle (LNP) for delivery to hepatocytes (Zhang et al., 2019, 2020b). Different from conventional siRNAs that are usually projected to target coding sequence regions, patisiran is designed to follow miRNA mechanisms by selectively binding to a conserved sequence in the 3′-untranslated regions (UTR) of mutant and wild-type TTR mRNA, leading to a reduction of circulating TTR protein levels and accumulation in tissues. Clinical studies demonstrated the benefits of patisiran (0.3 mg/kg every 3 weeks, i.v. infusion) for the treatment of patients with hATTR amyloidosis, as indicated by a decrease of the modified Neuropathy Impairment Score +7 from baseline to month 18 among the patisiran treatment group compared with a steady increase in the placebo group (Adams et al., 2018). Meanwhile, overall incidence and types of adverse events did not differ in patisiran and placebo groups, suggesting that patisiran was tolerated in patients (Adams et al., 2018; Zhang et al., 2020b). It is also notable that, although both patisiran and inotersen act on the same molecular target for the treatment of the same disease, patisiran is administered less frequently and at much lower doses than inotersen (284 mg once weekly, s.c.), although their routes of administration are different (Table 2).

In November 2019, givosiran was the second siRNA drug approved by the FDA for the treatment of adults with acute hepatic porphyria (AHP) (Table 2) (de Paula Brandao et al., 2020; Scott, 2020). AHP is a rare, inherited, and life-threatening disease caused by disruption of hepatic heme biosynthesis. The accumulation of neurotoxic heme intermediates δ-aminolevulinic acid (ALA) and porphobilinogen (PBG) in patients leads to acute debilitating neurovisceral attacks and even disabling chronic symptoms (Sardh et al., 2019). Givosiran is a 19-bp, chemically modified siRNA (Table 2) whose sense and antisense sequences are 5′-MeC-MeA-MeG-MeA-MeA-MeA-fG-MeA-fG-MeU-fG-MeU-fC-MeU-fC-MeA-MeU-MeC-MeU-MeU-MeA-3′ and 5′-MeU-fA-fA-fG-MeA-fU-MeG-fA-MeG-fA-MeC-fA-MeC-fU-MeC-fU-MeU-fU-MeC-fU-MeG-MeG-MeU-3′, respectively (Me = 2′-O-methyl and f = 2′-fluoro). The sense sequence is covalently attached to a triantennary GalNAc ligand at the 3′ end for an improved internalization of GalNac-conjugated siRNAs into hepatocytes. Givosiran selectively binds to the mRNA of δ-ALA synthase 1 (ALAS1), a rate-limiting enzyme in hepatic heme biosynthesis that is responsible for the formation of ALA from succinyl-CoA and glycine, to induce gene silencing, which subsequently reduces ALA and PBG levels and lessens factors associated with attacks and other

symptoms of AHP (de Paula Brandao et al., 2020; Sardh et al., 2019). Although the results of a phase III clinical trial have not been published yet, a phase I study showed that monthly subcutaneous administration of 2.5 mg/kg givosiran to patients with AHP sharply decreased the *ALAS1* mRNA levels and returned ALA and PBG levels to near normal, and it subsequently led to a 79% lower mean annualized attack rate than the placebo group (Sardh et al., 2019). The approval of givosiran also highlights the utility of a GalNAc-based delivery system for the development of RNA therapeutics for the treatment of hepatic diseases.

The very recent approval of two siRNA drugs by the FDA provides incentives to develop novel siRNA therapeutics, and many are now under active clinical trials (https://www.clinicaltrials.gov/) (Yu et al., 2019). Lumasiran, or ALN-GO1, is a GalNAc-conjugated investigative siRNA drug for the treatment of primary hyperoxaluria type 1, an inherited rare disease arising from disruption of glyoxylate metabolism (Liebow et al., 2017). Preclinical studies have demonstrated that, through selective targeting of the mRNA of hydroxyacid oxidase (glycolate oxidase) 1, lumasiran administered subcutaneously was able to reduce oxalate production in multiple animal models (Liebow et al., 2017). Presently, an open-label phase III clinical trial (NCT03905694) is underway for the investigation of the efficacy, safety, PK, and PD of lumasiran among infants and young children with primary hyperoxaluria type 1. Inclisiran, or ALN-PCSSC, is another synthetic siRNA with extensive chemical modifications and is covalently connected to a triantennary GalNAc ligand that is designed to target the mRNA of hepatic proprotein convertase subtilisin kexin type 9. Previous clinical studies consistently demonstrated the effectiveness of inclisiran in reducing the plasma proprotein convertase subtilisin kexin type 9 and LDL cholesterol levels in healthy individuals and patients at high risk for cardiovascular disease who had elevated LDL cholesterol levels (Fitzgerald et al., 2014, 2017; Ray et al., 2017). Moreover, a single-dose treatment with inclisiran was shown to cause a durable reduction in LDL cholesterol levels among the subjects over 1 year (Ray et al., 2019), and inclisiran showed similar efficacy and safety profiles among individuals with normal and impaired renal functions (Wright et al., 2020). A double-blind, placebo-controlled, and open-label phase II/III study (NCT03851705) is ongoing to evaluate the safety and efficacy of inclisiran in patients with HoFH. In addition, fitusiran (or ALN-AT3SC) is an investigative siRNA drug that is designed to suppress the production of antithrombin (encoded by the gene serpin family C member 1 (*SERPINC1*) through selective interference with SERPINC1 mRNA in the liver for the treatment of hemophilia A and B, inherited bleeding disorders arising from impaired thrombin production (Machin and Ragni, 2018). Administered subcutaneously once monthly, fitusiran was shown to reduce plasma antithrombin levels

in a dose-dependent manner and increase thrombin production in patients with hemophilia A or B who did not have inhibitory alloantibodies (Pasi et al., 2017). Presently, two phase III clinical trials (NCT03549871 and NCT03754790) are underway to evaluate the efficacy and safety of fitusiran among patients with hemophilia A and B.

siRNA drugs under clinical development are also expanded to other therapeutic areas, including oncology, that impact millions of patients. For instance, siG12D-LODER is an siRNA that specifically targets the Kirsten rat sarcoma viral oncogene homolog (KRAS) mutant G12D mRNA, a driver oncogene present in various types of cancers, especially pancreatic cancer (Zorde Khvalevsky et al., 2013; Golan et al., 2015; Ramot et al., 2016). A previous phase I clinical study showed that combination treatment with siG12D-LODE and gemcitabine was well tolerated, and potential efficacy among patients with locally advanced pancreatic cancer was shown (Golan et al., 2015). As such, siG12D-LODER is currently under a phase II clinical study to evaluate its efficacy, safety, tolerability, and PK for the treatment of patients with unresectable, locally advanced pancreatic cancer, as combined with standard chemotherapy (i.e., gemcitabine plus nanoparticle albumin-bound paclitaxel) in comparison with chemotherapy alone. As another example, synthetic siRNA targeting a protein-tyrosine kinase named ephrin type-A receptor 2 (EphA2) was shown to be effective in controlling tumor growth in xenograft mouse models (Landen et al., 2005). Furthermore, EphA2-siRNA encapsulated with 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine nanocomplex at tested doses was tolerated in murine and primate models (Wagner et al., 2017). A phase I clinical trial (NCT01591356) is now recruiting patients with advanced solid tumors to evaluate the safety and toxicity profiles of an 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine–encapsulated, EphA2-targeted siRNA drug administered intravenously.

3. MicroRNAs. MicroRNAs are a superfamily of genome-derived small ncRNAs governing post-transcriptional gene regulation that was first discovered in *Caenorhabditis elegans* in 1993 (Lee et al., 1993; Wightman et al., 1993). More than 1900 miRNAs have been identified in humans (Kozomara and Griffiths-Jones, 2014; Kozomara et al., 2019). Canonical biogenesis of miRNAs starts from the transcription of miRNA-coding sequences by RNA polymerase II to long primary miRNA (pri-miRNA) transcripts within the nucleus (Lee et al., 2004). The pri-miRNA is then cleaved to shorter precursor miRNA (pre-miRNA) by the RNase III Drosha complexed with RNA-binding protein DiGeorge syndrome chromosome region 8 (Lee et al., 2003; Denli et al., 2004; Gregory et al., 2004; Han et al., 2004). After being transported into the cytoplasm by RAS-related nuclear protein-GTP–dependent exportin-5 (Bohnsack et al., 2004; Lund et al., 2004), pre-miRNAs are cleaved, double-stranded

miRNA duplexes by cytoplasmic RNase III Dicer complexed with transactivation-responsive RNA-binding protein (Hutvágner et al., 2001; Lee et al., 2002; Zhang et al., 2004a; Haase et al., 2005). The miRNA duplex associated with miRNA-induced silencing complex is then unwound to offer two strands, among which the guide strand mature miRNA binds to the target mRNA through partial complementary base pairings, with corresponding miRNA response element usually present within the 3′UTR, leading to translational inhibition or transcript degradation or cleavage (Hutvágner and Zamore, 2002; Bagga et al., 2005; Pillai et al., 2005; Petersen et al., 2006). On the other hand, functional miRNAs may be generated through noncanonical pathways, such as those pre-miRNAs directly excised from introns not dependent upon Drosha (Okamura et al., 2007; Ruby et al., 2007), Dicer-independent miRNAs (Cheloufi et al., 2010; Cifuentes et al., 2010; Ho et al., 2018) 3), and miRNAs or small RNAs (sRNAs) derived from small nucleolar RNAs (Ender et al., 2008; Pan et al., 2013) or transfer RNAs (tRNAs) (Maute et al., 2013; Kuscu et al., 2018).

One miRNA can simultaneously regulate the expression of multiple transcripts, since the recognition of a target mRNA by miRNA does not require perfect base pairing. Through the control of multiple genes involved in the same biological processes, many miRNAs have been shown to play important roles in pathogenesis of human diseases, including lethal cancers [for reviews, see Ambros (2004), Bader et al. (2010), Esteller (2011), Rupaimoole and Slack (2017)]. In addition, compared with normal tissues, there is generally a decrease or loss of oncolytic miRNAs (e.g., let-7a/b/c-5p, miR-34a-5p and miR-124-3p) and overexpression of oncogenic miRNAs (e.g., miR-21-5p) in tumor tissues and carcinoma cells [for reviews, see Esteller (2011), Rupaimoole and Slack (2017)], although there are some exceptions. Therefore, some tumor-suppressive miRNAs lost in cancer cells may be restored, e.g., by using synthetic miRNA mimics and virus- or plasmid-based expression, and oncogenic miRNAs overexpressed in cancer cells may be inhibited, e.g., with chemically synthesized single-stranded ASO (termed antagomirs or miRNA inhibitors) for the control of tumor progression (Ho and Yu, 2016; Petrek and Yu, 2019; Yu et al., 2019). The "miRNA replacement therapy" strategy is of particular interest, compared with "antagonism" of oncogenic miRNAs, because miRNAs are endogenous components and reintroduced miRNAs may be well tolerable in cells. The premise of an miRNA replacement therapy strategy has been demonstrated by many preclinical studies (Bader et al., 2010; Rupaimoole and Slack, 2017; Petrek and Yu, 2019) that are amendable to clinical investigations (Beg et al., 2017; Hong et al., 2020).

Human miR-34a-5p is one of the most promising miRNAs for replacement therapy, with well established tumor-suppressive functions while downregulated in a wide range of solid tumors [for reviews, see Bader (2012),

Yu et al. (2019)]. Through effective interference with various oncogenes underlying tumor progression and metastasis, the efficacy of miR-34a-5p was consistently documented in many types of xenograft tumor mouse models (Wiggins et al., 2010; Liu et al., 2011; Pramanik et al., 2011; Craig et al., 2012; Kasinski and Slack, 2012; Wang et al., 2015b; Zhao et al., 2015, 2016; Jian et al., 2017; Ho et al., 2018). Therefore, a liposome-encapsulated synthetic miR-34a mimic, MRX34, became the first miRNA entering phase I clinical trial for the treatment of advanced solid tumors, including unresectable liver cancer (Beg et al., 2017; Hong et al., 2020). As the maximum tolerated dose was revealed to be 110 mg/m^2 in patients without hepatocarcinoma, a high incidence of adverse events (e.g., 100% all grades and 38% grade 3 among all patients), such as fever, fatigue, back pain, nausea, diarrhea, anorexia, and vomiting, was found among patients with liver cancer receiving treatment with MRX34 (10–50 mg/m^2, i.v., biweekly) who required palliative management with dexamethasone premedication. Nevertheless, MRX34 did exhibit antitumor activity among patients with refractory advanced solid tumors (Beg et al., 2017; Hong et al., 2020), offering valuable insight into the development of miRNA therapeutics.

One randomized, double-blind, phase I/II clinical trial (NCT04120493) is underway to explore the safety, tolerability, and efficacy signals of multiple ascending doses of striatally administered, adenoassociated viral vector–carried miR-155, which targets the total huntingtin (HTT) transcripts—namely, rAAV5-miHTT or AMT-130—in early manifest Huntington disease (HD). This strategy is based on previous findings on the efficacy and safety of miHTT therapy in preclinical models, including the use of an Hu128/21 HD mouse model (Miniarikova et al., 2016), acute HD rat model (Miniarikova et al., 2017), transgenic HD minipig model (Evers et al., 2018), neuronal and astrocyte cells derived from patients with HD (Keskin et al., 2019), and humanized Hu128/21 mouse model of HD (Caron et al., 2020).

Although there is no single miRNA drug that has yet to be approved by the FDA for medical use, the first siRNA drug, patisiran, seems to mimic miRNA mechanisms of action aforementioned, i.e., through selective binding to the 3′UTR of TTR mRNA. In addition, many studies have been conducted, and others are still underway, for the identification of miRNAs as potential diagnostic or prognostic biomarkers for patients with particular diseases or treatments (Hayes et al., 2014; Kreth et al., 2018; Pogribny, 2018), besides the use of miRNAs as interventional agents.

4. RNA Aptamers. Aptamers are single-stranded, highly structured DNA or RNA oligonucleotides that can bind to a wide variety of molecular targets, including proteins, peptides, DNAs, RNAs, small molecules, and ions, with high affinity and specificity. Upon binding to target protein, RNA aptamer behaves like a nucleic acid antibody or chemical inhibitor to modulate protein function (Fig. 1) for the control of disease (Bunka and Stockley, 2006; Bouchard et al., 2010; Kaur et al., 2018). Actually, natural RNA-protein complex was first identified in bacteria, among which the RNA molecule is an essential component for the activity of RNase P complex in the processing of precursor tRNA into active tRNA, which could also be inhibited by various RNAs or aptamers (Stark et al., 1978; Kole and Altman, 1979). There is also autocatalytic RNA or ribozyme, which undergoes self-splicing upon binding with monovalent and divalent cations (Kruger et al., 1982). Highly structured RNA elements, or "aptamers," are also present within human immunodeficiency virus (HIV)-1 to interact with target proteins for gene expression and viral replication (Feng and Holland, 1988; Marciniak et al., 1990). Moreover, intrinsic RNAs or riboswitches can sense small-molecule metabolites and then control target gene expression (Mironov et al., 2002; Nahvi et al., 2002; Winkler et al., 2002a). Ligand binding ribozymes and riboswitches have been identified in humans, as well (Salehi-Ashtiani et al., 2006; Ray et al., 2009).

With the understanding of the interactions of functional RNAs with proteins as well as other ligands, a high-throughput technology, systematic evolution of ligands by exponential enrichment, was also developed for the identification and development of selective and potent RNA aptamers or ribozymes (Ellington and Szostak, 1990; Robertson and Joyce, 1990; Tuerk and Gold, 1990). Chemical modifications of selected RNA aptamers may increase metabolic stability and improve PK properties, similar to ASOs and siRNAs (Khvorova and Watts, 2017; Yu et al., 2019, Ho and Yu 2016). Furthermore, mirror-image *L*-ribonucleic acids resistant to degradation by RNases have been used for the synthesis and development of artificial aptamers called Spiegelmer (Vater and Klussmann, 2015).

In 2004, pegaptanib was the first RNA aptamer drug approved by the FDA for the management of neovascular age-related macular degeneration (AMD) (Table 2) (Gryziewicz, 2005), supporting the utility of aptamers to interfere with protein targets for the control of human diseases (Fig. 1).

AMD is a leading cause of low vision in the elderly in developed countries, and neovascular AMD, accounting for approximately 10% of all forms, is responsible for 90% of the severe loss of vision (Gragoudas et al., 2004). With the understanding of the role of vascular endothelial growth factor (VEGF) in pathogenesis of neovascular AMD, ocular VEGF has become an attractive target for the treatment of neovascular AMD (Hubschman et al., 2009; Miller, 2019). Pegaptanib, a 28-nt RNA aptamer (Table 2) with a sequence 5′-fC-MeG-MeG-A-A-fU-fC-MeA-MeG-fU-MeG-MeA-MeA-fU-MeG-fC-fU-fU-MeA-fU-MeA-fC-MeA-fU-fC-fC-MeG-3′-3′-dT-5′ and

covalently linked to two branched 20-kDa polyethylene glycol moieties was designed to selectively bind and block the activity of extracellular VEGF—in particular, the 165-amino-acid isoform ($VEGF_{165}$) (Gragoudas et al., 2004). The benefits of pegaptanib in improving visual acuity were demonstrated in patients with neovascular AMD, and intravitreal injection could induce some potentially modifiable risk of adverse events (Gragoudas et al., 2004; Gonzales, 2005). Nevertheless, the market share of pegaptanib declined since 2011 because of competition from anti-VEGF antibody drugs such as ranibizumab and bevacizumab (Yin and Rogge, 2019).

Olaptesed pegol (NOX-A12), a pegylated 45-nt RNA Spiegelmer designed to selectively target the small chemokine stromal cell-derived factor 1 or C-X-C motif chemokine 12 with high affinity, was effective at preventing the binding of stromal cell–derived factor 1 to its receptors CXC receptor 4 and CXC receptor 7 and thus inhibiting the subsequent signal transduction to achieve control of angiogenesis and metastasis, as well as improvement of other anticancer therapies (Roccaro et al., 2014; Deng et al., 2017). Two phase IIa studies showed that patients with relapsed/refractory multiple myeloma (Ludwig et al., 2017) and chronic lymphocytic leukemia (Steurer et al., 2019) were highly responsive to olaptesed pegol therapy in combination with bortezomib-dexamethasone and bendamustine-rituximab, respectively. One active clinical trial (NCT04121455) is underway to evaluate the safety and efficacy of olaptesed pegol in combination with irradiation among patients with inoperable or partially resected first-line glioblastoma. Another pegylated Spiegelmer, lexaptepid pegol (NOX-94), binds to human hepcidin with high affinity and thus inhibits its biological function (Schwoebel et al., 2013) for the treatment of anemia of chronic disease. A first-in-human study (NCT01372137) showed that lexaptepid pegol was able to inhibit hepcidin and dose dependently elevate serum iron and transferrin saturation, and it was generally safe and tolerated in healthy subjects, with mild and transient transaminase increases at higher doses (Boyce et al., 2016). After additional investigations in patients (e.g., NCT02079896), no clinical trial is currently open to evaluate the safety and efficacy of lexaptepid pegol. Although clinical development of new aptamer drugs seems less active in recent years, there is growing interest in developing aptamers for drug delivery and as diagnostic agents (Bouvier-Müller and Ducongé, 2018; Kaur et al., 2018).

5. Messenger RNAs. The development and use of mRNAs as a novel class of drug modalities has great potential in vaccination, protein replacement therapy, and antibody therapy for the treatment of a wide variety of human diseases, including infections, cancers, and genetic disorders (Sahin et al., 2014; Weissman and Kariko, 2015; Pardi et al., 2018; Kowalski et al., 2019). This concept was first demonstrated by the findings on efficient expression of target proteins in mouse tissues in vivo after the administration of in vitro–transcribed (IVT) mRNAs, which was reported in 1990 (Wolff et al., 1990). After extensive preclinical studies, many IVT mRNA therapeutics have already entered clinical trials (Heiser et al., 2002; Weide et al., 2009; Rittig et al., 2011; Allard et al., 2012; Van Gulck et al., 2012; Maus et al., 2013; Wilgenhof et al., 2013; Bahl et al., 2017; Leal et al., 2018; de Jong et al., 2019; Papachristofilou et al., 2019). Different from plasmid DNA or virus-based gene therapy, mRNA drugs are translated into target proteins by the cellular machinery without interference with the genome (Fig. 1). To ensure translation ability and efficiency, an mRNA needs to contain not only the whole open reading frame of target protein but also intact 5′ and 3′UTRs as well as 5′ cap and 3′ poly(A) tail. Therefore, the mRNA drug molecule is much bigger than other types of RNA therapeutics (Fig. 1). Likewise, direct administration of mRNA therapeutics to patients requires efficient delivery systems to protect mRNAs from degradation by RNases and cross-cellular barrier in vivo. Alternatively, patients may be treated with autologous transplantation of T cells or dendritic cells (DCs) that are reprogrammed with mRNA drugs ex vivo.

Because of the high sensitivity of immune cells in recognizing antigens that can be coded by exogenous mRNAs, as well as their intrinsic immune-stimulatory effects (Hoerr et al., 2000; Weissman et al., 2000; Fotin-Mleczek et al., 2011), mRNA therapeutics hold great promise as vaccines for the treatment of infectious and cancerous diseases. For instance, a phase I study revealed the effectiveness and safety of autologous transplantation of DCs transfected with mRNA encoding prostate-specific antigen in the induction of prostate-specific antigen–specific immunity and impact on surrogate clinical endpoints among patients with metastatic prostate cancer (Heiser et al., 2002). Another phase I/II study showed the impact of intradermal injection of protamine-formulated mRNAs coding multiple tumor-associated antigens, e.g., melan-A, tyrosinase, glycoprotein 100, melanoma-associated antigen (Mage)-A1, Mage-A3, and survivin, in patients with metastatic melanoma (Weide et al., 2009). A very recent phase Ib clinical trial also established the benefits of immunotherapy consisting of protamine-protected, sequence-optimized mRNA (BI1361849 or CV9202) encoding six non–small-cell lung cancer (NSCLC)-associated antigens [New York Esophageal Squamous Cell Carcinoma-1 (NY-ESO-1), MAGE-C1, MAGE-C2, survivin, 5T4, and Mucin-1] among patients with stage IV NSCLC (Papachristofilou et al., 2019). As such, a phase I/II study (NCT03164772) is underway to evaluate the safety and efficacy of combination therapy with CV9202 mRNA vaccine and checkpoint inhibitors (e.g., anti–programmed death-ligand 1 durvalumab and anti–cytotoxic T-lymphocyte antigen 4 tremelimumab) for the treatment of NSCLC. Other ongoing clinical trials include a dose-escalation and efficacy study of intratumoral administration of LNP-encapsulated

mRNA-2416 encoding human OX40L (NCT03323398) and mRNA-2752 encoding human OX40L, IL-23, and IL-36γ (NCT03739931), alone or combined with durvalumab, for patients with advanced malignancies.

Managing infectious diseases through mRNA vaccine is also actively assessed in a clinical setting. One investigation demonstrated that autologous transplantation of monocyte-derived DCs pretreated with mRNAs encoding Group-specific antigen and a chimeric transactivating regulatory protein (Tat)-anti-repression trans-activator (Rev)-negative regulatory factor (Nef) protein was tolerated and effective at enhancing antiviral responses in six patients infected with HIV-1 and under stable and highly active antiretroviral therapy (Van Gulck et al., 2012). Another phase I/IIa study showed that vaccinations with autologous DCs electroporated with mRNA encoding Tat-Rev-Nef were well tolerated and able to induce vaccine-specific immune responses among 17 HIV-1–infected patients who were stable on combined antiretroviral therapy, after which combined antiretroviral therapy was interrupted (Allard et al., 2012). The benefits of this vaccination therapy with mRNA-transfected DCs were further demonstrated by very recent clinical studies (Gandhi et al., 2016; Leal et al., 2018; de Jong et al., 2019); however, the exact mRNA vaccines are different. Furthermore, clinical studies revealed a robust prophylactic immunity of LNP-carried, specifically modified mRNA vaccines encoding hemagglutinin proteins of avian influenza virus A H10N8 induced in humans, although some mild to severe adverse events were noted (Bahl et al., 2017). In addition, a randomized, observer-blind, placebo-controlled, and dose-ranging phase I study (NCT04064905) is recruiting healthy flavivirus seropositive and seronegative adults for the evaluation of the safety and immunogenicity of a Zika vaccine (mRNA-1893).

To combat against the ongoing global severe acute respiratory syndrome coronavirus (SARS-CoV)-2 pandemic, or coronavirus disease 2019 crisis, which as of the acceptance of this paper for publication, has caused more than 17 million confirmed cases and over 673,000 deaths worldwide and more than 4.6 million cases and over 154,000 deaths in the United States (https://www.worldometers.info/coronavirus/), enormous efforts are underway to develop treatment and preventive strategies, including mRNA vaccines (Corey et al., 2020; Yi et al., 2020a). The SARS-CoV-2 virus belongs to a family of positive-sense, single-stranded RNA coronaviruses whose replication depends on the translation of viral RNA into proteins and reproduction of viral RNAs, as well as assembly of the capsid within host cells, after the interactions between viral spike proteins and host cellular membrane proteins (Hoffmann et al., 2020; Letko et al., 2020). Given the important role in SARS-CoV-2 viral infection, the spike proteins have emerged as potential targets for the development of small-molecule and protein drugs as well as vaccines. Indeed,

one LNP-encapsulated mRNA vaccine encoding a 103 transmembrane–anchored SARS-CoV-2 spike protein with the native furin cleavage site (mRNA-1273) has quickly entered into clinical trials (NCT04405076 and NCT04283461), attributable to the structure-guided design of target protein/mRNA, fast LNP/mRNA vaccine platform technology, and some promising preclinical observations (K. S. Corbett et al., preprint, https://doi.org/10.1101/2020.06.11.145920). Although the unprecedented need for vaccination against SARS-CoV-2 is clear, establishing the safety and efficacy of a vaccine takes time before it can be used to immunize a large population to protect global public health.

IVT mRNAs encoding target proteins and antibodies may be developed for protein replacement therapy and antibody therapy, respectively. This is an alternative strategy to classic gene therapy using DNA materials, protein/antibody molecules, and the most recent gene editing technology for the treatment of monogenic disorders caused by impaired or disrupted protein synthesis in body (Martini and Guey, 2019), as well as some common diseases such as infection and cancer (Schlake et al., 2019). As an example, methylmalonic acidemia (MMA) is an inherited metabolic disorder usually found in early infancy that ranges from mild to life-threatening, and about 60% of MMA cases are attributed to the deficiency of hepatic methylmalonyl CoA mutase (MUT) synthesis, caused by mutations in the *MUT* gene. A pseudouridine-modified, codon-optimized mRNA encoding human MUT formulated with LNP has been developed as mRNA replacement therapy for the treatment of MMA, and its efficacy and safety profiles have been established very recently in murine models (An et al., 2017, 2019). Currently, an open-label, dose-escalation phase I/II clinical study (NCT03810690) is underway to evaluate the safety, PK, and PD of mRNA-3704 encoding functional MUT enzyme among patients with isolated MMA due to MUT deficiency between 1 and 18 years of age with elevated plasma methylmalonic acid.

With the discovery of gene editing technologies and development of novel therapeutic strategies, there is also growing interest in using mRNAs to introduce target proteins to achieve gene editing. They include the use of mRNAs encoding zinc finger nucleases (Geurts et al., 2009; Wood et al., 2011; Huang et al., 2014; Wang et al., 2015a; Conway et al., 2019), transcription activator–like effector nucleases (Tan et al., 2013; Wefers et al., 2013; Poirot et al., 2015; Nanjidsuren et al., 2016), transposases (Wilber et al., 2006; Ivics et al., 2014a,b; Ellis et al., 2017), CRISPR-associated proteins, or endonucleases (e.g., Cas9 and Cas12a) (Wang et al., 2013; Wu et al., 2013; Yin et al., 2016; Ren et al., 2017; Cromer et al., 2018; Xu et al., 2018; Gurumurthy et al., 2019) to enable genome editing or alteration of specific gene sequences. Rather, the specificity and safety of editing a genome with such new modalities warrant more extensive and critical

studies, and their utility for the treatment of human diseases is mainly under preclinical investigations thus far.

6. Guide RNAs. The prokaryotic CRISPR/Cas immune system (Jansen et al., 2002; Makarova et al., 2006; Barrangou et al., 2007) has been developed as a novel and accessible technology to precisely edit genome sequence toward irreversible knockout or knockin of a target gene in mammalian cells and organisms (Jinek et al., 2012; Cong et al., 2013; Mali et al., 2013; Mashiko et al., 2013; Kim et al., 2017; Anzalone et al., 2019), as compared with RNAi, which does not completely eradicate gene expression, and mRNA therapy, which transiently introduces functional proteins. The CRISPR/Cas–based gene editing technology relies on two essential components, a designed gRNA and the RNA-guided Cas nuclease. Through its hairpin scaffold binding to Cas to form Cas-gRNA ribonucleoprotein (RNP) complex, the gRNA recognizes a protospacer-adjacent motif element and a 20-nucleotide sequence in the genome through complementary base pairings and thus directs the Cas nuclease to generate a double-stranded DNA break or a single-stranded break (nick) to achieve genome engineering. As such, the CRISPR/Cas technology has been actively evaluated toward the development of new therapies for the treatment of human diseases, including monogenetic disorders, infection, and cancer (Xue et al., 2014; Dever et al., 2016; Long et al., 2016; Nelson et al., 2016; Tabebordbar et al., 2016; Eyquem et al., 2017; Zhang et al., 2017; Georgiadis et al., 2018; Xu et al., 2019b).

Different from other types of RNA therapeutics, the success of CRISPR/Cas–based genome editing and therapy relies not only on exogenous gRNA but also foreign Cas nuclease, that latter of being a large protein around 160 kDa in size. Moreover, both the gRNA and Cas9 protein need to get into the nucleus and form RNP to exercise genome editing (Fig. 1). Besides conventional intracellular expression using plasmid DNA (pDNA) or virus vector–based materials, the gRNAs may be produced by IVT or chemical synthesis and directly introduced into cells or organisms with particular delivery systems, alone or combined with Cas nuclease as RNP. Each approach has its own advantages and disadvantages regarding the cost, stability, efficiency, specificity, and safety [for reviews, see Sahel et al. (2019), Chen et al. (2020)]. With the knowledge of chemical modifications in protecting against RNase digestion and avoiding immunogenicity, chemically modified gRNAs have been shown to enhance genome editing efficiency and target specificity in mammalian cells (Hendel et al., 2015; Rahdar et al., 2015; McMahon et al., 2018). Thus, chemoengineered gRNAs are expected to improve the development of CRISPR/Cas–based therapies.

Multiple clinical trials have been launched to investigate the safety and effectiveness of CRISPR/Cas–based therapies (https://www.clinicaltrials.gov/), but no results are reported yet. The first clinical trial involving CRISPR/Cas gene editing (NCT02793856), open in 2016, was a dose-escalation study on autologous implantation of programmed cell death protein 1 (PD-1; coded by *PDCD1* gene) knockout T cells for the treatment of patients with advanced NSCLC that has progressed after all standard treatments. Immune checkpoint regulator PD-1 is a membrane receptor responsible for the inhibition of T cell activation, thereby decreasing autoimmune reactions and allowing immune escape of cancers. Antibodies against PD-1 or its ligand have been successfully used for the treatment of various types of cancers, and CRISPR/Cas–based PD-1 immunotherapy represents a novel strategy to combat cancer. Another phase I trial (NCT03399448) was initiated in 2018 to define the safety profile of NY-ESO-1 redirected autologous T cells with CRISPR-edited endogenous T cell receptor (TCR) and PD-1 autologous T cells, in particular, transduced with a lentiviral vector to express cancer/ testis antigen 1 or NY-ESO-1 and electroporated with CRISPR gRNA to disrupt expression of endogenous T cell receptor TCRα and TCRβ, as well as PD-1 (NYCE T Cells), among subjects with a confirmed diagnosis of relapsed refractory multiple myeloma, melanoma, synovial sarcoma, or myxoid/round cell liposarcoma. Presently, there are a number of phase I/II studies on the safety and efficacy of autologous CD34+ human hematopoietic stem and progenitor cells modified with CRISPR/Cas at the erythroid lineage-specific enhancer of the B-cell lymphoma/leukemia 11A gene (CTX001) in patients with transfusion-dependent β-thalassemia (NCT03655678) and patients with severe sickle cell disease (NCT03745287). In addition, the safety and efficacy of CD19-directed T cell immunotherapy comprising allogeneic T cells modified with CRISPR/Cas (CTX110) for the treatment of patients with relapsed or refractory B cell malignancies are currently under clinical evaluation (NCT04035434).

7. Other Forms of RNAs. There are also efforts devoted to develop other forms of RNAs for the treatment of human diseases, such as short or small hairpin RNAs (shRNAs) (Brummelkamp et al., 2002; Paddison et al., 2002), ribozymes or catalytic RNAs (Burnett and Rossi, 2012), and circular RNAs (Holdt et al., 2018; Santer et al., 2019). Like siRNAs and miRNAs, shRNAs can be used to achieve selective gene silencing effects via the RNAi mechanism. They are usually introduced into cells through viral vectors or pDNA, and shRNAs can be chemically synthesized with desired modifications. Similar to a pre-miRNA in size and with hairpin structure, shRNA follows the miRNA biogenesis pathway once shRNA precursor is transcribed from the coding sequence integrated into the host genome in the nucleus. The guide strand derived from shRNA in the cytoplasm is loaded into the RISC to silence target gene expression in the same manner as synthetic siRNAs. Likewise, there are some ongoing clinical studies on the

benefits of new modalities involving shRNAs (https://www.clinicaltrials.gov/). For instance, an open-label phase I study (NCT03282656) is recruiting patients with sickle cell disease to evaluate the feasibility of autologous bone marrow–derived CD34+ HSC cells transduced with the lentiviral vector containing an shRNA targeting B-cell lymphoma/leukemia 11A. As another example, a plasmid named pbi-shRNA Ewing sarcoma breakpoint region 1 (EWS)/Friend leukemia integration 1 transcription factor (FLI1) was developed to target the *EWS/FLI1* fusion gene, which is a driver in the pathogenesis and maintenance of Ewing's sarcoma (Rao et al., 2016). Formulated with lipoplex (LPX), the pbi-shRNA EWS/FLI1 LPX was found effective in type 1 Ewing's sarcoma xenograft mouse models (Rao et al., 2016), leading to the opening of a phase 1 clinical study (NCT02736565) on pbi-shRNA EWS/FLI1 LPX in patients with advanced Ewing's sarcoma.

Ribozymes are a specific group of RNA molecules that are able to catalyze biochemical reactions (Kruger et al., 1982). The hammerhead or hairpin structures facilitate a ribozyme to cleave target RNAs in specific sequences, and the substrate recognition domain of the ribozyme can be artificially engineered to stimulate site-specific cleavage in cis (the same nucleic acid strand) or trans (a noncovalently linked nucleic acid) (Scherer and Rossi, 2003). Moreover, ribozymes are amenable to in vitro selection or evolution, e.g., by systematic evolution of ligands by exponential enrichment approaches (Ellington and Szostak, 1990; Robertson and Joyce, 1990; Tuerk and Gold, 1990), toward improved properties or new functions for therapeutic and diagnostic purposes. As with enzymes, catalytic RNAs often require cofactor magnesium ions to exert biotransformations (Ban et al., 2000). The development of ribozymes as therapeutic molecules has been largely dependent on the improvement of PK properties via chemical modifications (Burnett and Rossi, 2012). Indeed, a chemoengineered, antiangiogenic ribozyme against the oncogene fms-like tyrosine kinase (FLT-1) by targeting the vascular endothelial growth factor receptor-1 mRNA was effective at cleaving FLT-1/vascular endothelial growth factor receptor-1 mRNA and dose dependently inhibiting lung metastasis in an animal model (Pavco et al., 2000). Although anti–FLT-1 ribozyme (RPI.4610 or Angiozyme) was well tolerated among patients in both phase I and II studies (Kobayashi et al., 2005; Morrow et al., 2012), there was a lack of clinical efficacy for the treatment of patients with metastatic breast cancer, precluding RPI.4610 from further development (Morrow et al., 2012). In addition, a phase II clinical study (NCT01177059) was conducted to evaluate the potential benefits of an anti–HIV-1 ribozyme (OZ1) for the treatment of patients with HIV-1 infection; however, the results have not been published yet.

C. Challenges in the Development of RNA Drugs

Although they offer an unprecedented opportunity to expand the range of druggable targets for the control of potentially all kinds of human diseases, there were only fewer than 10 RNA drugs approved by the FDA over the past 2 decades (Table 2). Most RNA drugs are designed to act on intracellular pharmacological targets (Fig. 1), whereas RNA molecules are intrinsically unstable and are unable to freely cross cellular membranes, unlike traditional small-molecule and protein medications (Table 1). In addition, RNA drugs may not be highly selective toward their targets, as expected. Indeed, exogenous RNAs are commonly recognized by cellular defense systems, which could lead to acute immune response, cytokine release syndrome, or even severe cytokine storms. Therefore, the development of efficacious and safe RNA therapeutics has proven to be highly challenging.

1. Choice of RNA Substances. The chemical nature of RNA molecules makes them highly susceptible to ubiquitous RNases (Houseley and Tollervey, 2009), and chemically modified RNA analogs dominate RNA drug discovery and development. Indeed, a wide variety of chemical modifications may be introduced into an RNA molecule, the major strategy to improve RNA metabolic stability and PK properties (Bramsen and Kjems, 2012; Khvorova and Watts, 2017; Yu et al., 2019). For example, the change of PO linkage to PS makes the resulting RNA analog resistant to RNase degradation, and its PK properties can be further improved when its 2′-hydroxyl group on ribose is protected or directly substituted with fluorine. This approach has found ultimate success in the development of sRNA drugs such as ASOs, siRNAs, miRNAs, and aptamers, as all RNA drugs approved by the FDA thus far are chemoengineered RNA analogs (Table 2), supporting the utility of chemical modifications. Additionally, although it becomes much more expensive to chemically synthesize and modify longer RNAs, those chemoengineered molecules, such as gRNAs, can exhibit greater metabolic stability and biological function in human cells (Hendel et al., 2015; Rahdar et al., 2015).

The mRNA drugs are usually much bigger in size than other sRNAs used for gene silencing or genome editing (Fig. 1). In addition to the entire open reading frame of encoded protein, the therapeutic mRNA needs to contain the complete 5′ and 3′UTRs as well as 5′ cap and 3′ poly(A) tail to ensure an efficient translation within cells. Therefore, mRNA drug molecules are generally produced by a conventional IVT method using the T7 or SP6 RNA polymerase (Milligan et al., 1987; Beckert and Masquida, 2011). Compared with chemical synthesis, IVT represents an efficient and economic approach to generating a large therapeutic mRNA molecule that consists of essential components for intracellular translation. Although IVT is unable to specifically assemble post-transcriptionally modified or natural nucleosides into an mRNA molecule at particular sites, and the effects of such modifications on the efficiency of intracellular translation remain obscure, systemic incorporation

of pseudouridine (Karikó et al., 2008) or N1-methyl-pseudouridine (Svitkin et al., 2017) into mRNA substances could be achieved by IVT reactions, leading to an improvement of translational capacity and biological stability, as compared with unmodified counterparts.

The primary sequence, particularly a series of nucleobases, is critical for the RNA molecule to act on its RNA or DNA therapeutic target. This notion is also supported by the successful marketing of PMO drugs (Table 2), in which nucleobases are linked by morpholino phosphorodiamidate bonds. However, RNA secondary (e.g., helices or stems, loops, and bulges), tertiary (e.g., junctions, pseudoknot, and motifs), and quaternary (e.g., complexes) structures formed by Watson-Crick complementary base pairings and/or other types of physicochemical interactions (Butcher and Pyle, 2011; Jones and Ferré-D'Amaré, 2015; Schlick, 2018) are essential for its stability, plasticity, interactions with cofactors, function, and safety. There are also various types and unique post-transcriptional modifications (Limbach et al., 1994; Cantara et al., 2011; Yu et al., 2019) that are critical for the folding and functions of natural RNAs produced in living cells. In addition, post-transcriptional modifications have been shown to suppress immune responses in cells (Nallagatla et al., 2008; Gehrig et al., 2012), whereas many chemical modifications induce immunogenicity. Therefore, there are growing interests in developing bioengineering technologies to produce true biological RNA molecules in living cells for research and development (Ho and Yu, 2016; Yu et al., 2019, 2020), similar to protein research and drug development that create and use recombinant or bioengineered proteins.

Two novel approaches have been developed very recently to offer high-yield and large-scale fermentation production of bioengineered or biological RNA agents (BERAs), e.g., tens of milligrams target RNAs from 1 l of bacterial culture. One strategy involves the use of stable RNA carriers (Ponchon and Dardel, 2007; Ponchon et al., 2009; Li et al., 2014, 2015, 2018b; Chen et al., 2015; Ho et al., 2018), and the other method seeks direct overexpression in RNase III–deficient bacteria (Hashiro et al., 2019a,b). Among them, stable hybrid tRNA/pre-miRNA molecules have been identified and proven as the most robust and versatile carriers to accommodate various types of warhead RNAs, including miRNAs, siRNAs, aptamers, and other sRNAs (Chen et al., 2015; Wang et al., 2015b; Ho et al., 2018; Li et al., 2018b). This approach follows a similar workflow as protein bioengineering (Fig. 2). After a BERA/miRNA, siRNA, or sRNA substance of interest is designed, the corresponding coding sequence is cloned into a vector. Overexpression of target BERA in pDNA-transformed bacteria can be assessed by RNA gel electrophoresis analysis of total bacterial RNAs. Recombinant BERA may be isolated with different methods (e.g., anion exchange fast protein liquid chromatography), and the quality of purified BERA can be controlled by high-performance liquid chromatography (HPLC) analysis and endotoxin pyrogen testing (Chen et al., 2015; Ho et al., 2018; Petrek et al., 2019).

BERAs produced in living cells have been revealed to carry no or minimal post-transcriptional modifications (Ponchon and Dardel, 2007; Li et al., 2015; Wang et al., 2015b). Although naked BERAs are still susceptible to degradation by serum RNases, BERAs are readily
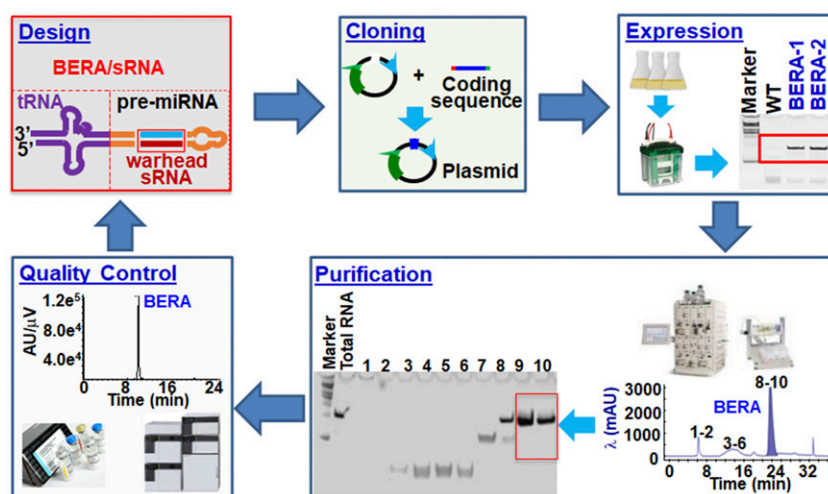
Fig. 2. The tRNA/pre-miRNA–based RNA bioengineering technology for the production of biological RNAi agents (BERA) for research and development. After the design of a target BERA/sRNA (e.g., miRNA, siRNA, and asRNA, etc.), the corresponding coding sequence is cloned into a vector. Expression of target BERA in bacterial culture is readily verified through RNA gel electrophoresis, and BERA can be purified to a high degree of homogeneity using different methods [e.g., anion exchange fast protein liquid chromatography (FPLC)]. Purity of isolated BERA is determined by HPLC analysis and endotoxin pyrogen testing. These bioengineered RNA molecules should better recapitulate the properties of natural RNAs to exert biological or pharmacological actions, as both are produced and folded in living cells. Indeed, BERA/sRNAs can be selectively processed to warhead sRNAs for the modulation of target gene expression in human cells and control of diseases in animal models. WT, wild type.

delivered into human carcinoma cells and xenograft tumor tissues by lipid or polymer-based materials; selectively processed to target warhead miRNAs or siRNAs to modulate target gene expression; and consequently inhibit cancer cell proliferation, tumor progression, and metastasis (Chen et al., 2015; Zhao et al., 2016; Jian et al., 2017; Jilek et al., 2017, 2019; Ho et al., 2018; Zhang et al., 2018; Li et al., 2019; Tu et al., 2019; Xu et al., 2019a; Yi et al., 2020b). In addition, these BERA-carried miRNAs and siRNAs have been shown to be equally or more effective than synthetic counterparts in the regulation of target gene expression and suppression of cancer cell growth (Chen et al., 2015; Wang et al., 2015b). These bioengineered RNA molecules should better recapitulate the properties of natural RNAs to exert structural, biological, or pharmacological actions, as both are produced and folded in living cells.

2. RNA Delivery Systems. A major challenge in the development of RNA drugs is to overcome the degradation by serum RNases and make RNAs to cross the membranes of targeted cells so that a sufficient number of RNA molecules can access intracellular targets to exert pharmacological effects (Fig. 3). As mentioned previously, some chemical modifications can greatly improve the metabolic stability and PK properties (Bramsen and Kjems, 2012; Ho and Yu, 2016; Khvorova and Watts, 2017; Yu et al., 2019) and thus make the resulting RNA substances more druggable. All the ASO therapeutics approved by the FDA carry extensive chemical modifications (Table 2). Without additional excipients, these ASOs can be distributed to targeted

cells to achieve efficacy, like small-molecule drugs. On the other hand, RNA drugs may be "actively delivered" to targeted cells or tissues through encapsulation/formulation with specific materials (Fig. 3) or carried by viral vectors, pDNAs, or intact cells (Tibbitt et al., 2016; Dowdy, 2017; Kaczmarek et al., 2017; Kowalski et al., 2019). The latter tends to switch the modalities to DNA/gene materials or engineered cells, whereas the former keeps the RNA substances as the active ingredients.

Lipids and lipid-like materials that resemble the lipid component of cell membrane and offer great biocompatibility are commonly used for the delivery of RNA agents besides peptides, hydrogels, dendrimers, and synthetic (e.g., polyamidoamine and polyethyleneimine) and natural polymers (e.g., chitosan) (Tibbitt et al., 2016; Kaczmarek et al., 2017). Similar to positively charged polymers or other substances, cationic lipids can electrostatically bind to RNAs to protect them against RNase cleavage and facilitate endocytosis. Interestingly, ionizable lipids that only become positively charged in acidic conditions have been shown to enhance endosomal release and reduce cytotoxicity (Kanasty et al., 2013). The successful use of LNP in RNA drug development is evident in the first FDA-approved siRNA drug, patisiran (Table 2). LNP is also used for the delivery of novel mRNA therapeutics, such as human MUT mRNA replacement therapy for the treatment of MMA, which has been evaluated in murine models (An et al., 2017, 2019) and entered clinical trials. In addition, LNP has been employed
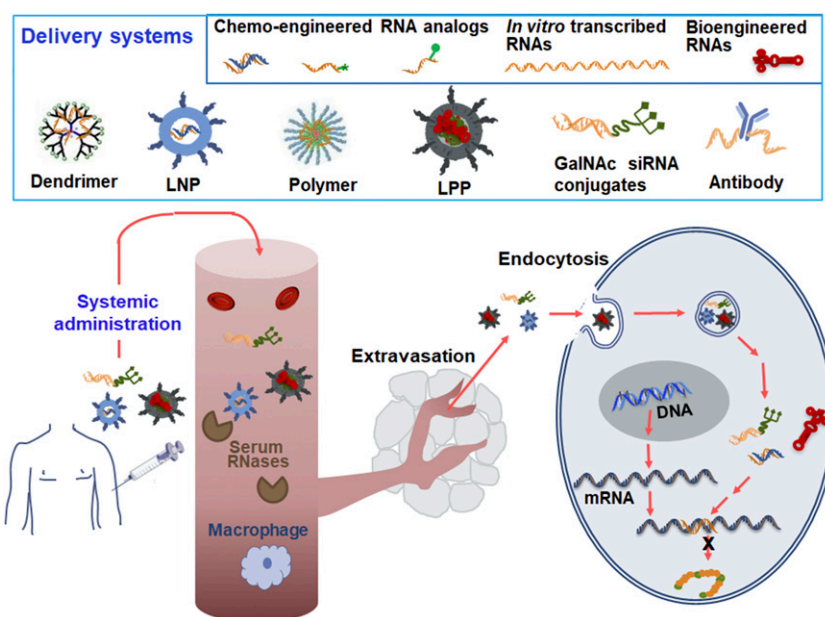
Fig. 3. Common approaches for the formulation and delivery of RNA therapeutics. RNA drugs administered intravenously need to be protected against excessive degradation by serum RNases and overcome the cell membrane barriers to gain access to intracellular targets. After entering cells through endocytosis or other mechanisms, RNA therapeutics are released in the cytoplasm, translocated, and incorporated into corresponding ribonucleoprotein complexes to silence target transcripts. As chemical modifications largely improve the metabolic stability and PK properties, RNA drug may be encapsulated in nanoparticle using different systems such as LNP and LPP. In addition, conjugation of a trivalent GalNAc to the RNA drug or use of antibody can improve the efficiency of delivery to targeted cells.

for the delivery of the gRNA-Cas9 RNP complex for genome editing (Wang et al., 2016).

To optimize LNP formulations, some hydrophobic moieties, such as cholesterol-lipid poly(ethylene glycol)-lipid, may be included to improve RNA delivery efficiency, and the ionizable property of LNP seems to be a determinant factor (Akinc et al., 2008; Love et al., 2010; Dahlman et al., 2014). Furthermore, as polyethylenimine-based polyplexes provide excellent delivery efficiency but pose a high risk of toxicity (Lv et al., 2006), lipidation of the polyethylenimine-RNA polyplex offers lipopolyplex (LPP), which may retain the benefits of both materials while reducing the toxicity of polyplex (Rezaee et al., 2016). Indeed, some recent studies have demonstrated that LPP showed more favorable biological properties for the delivery of nucleic acid agents (Schäfer et al., 2010; Ewe et al., 2014, 2017), including these novel BERAs produced and folded in living cells (Zhang et al., 2018; Jilek et al., 2019).

RNAs may be covalently conjugated to specific ligands, ranging from relatively smaller molecules (e.g., aptamers) to large molecules (e.g., lipids, peptides, and antibodies). Ligand-directed delivery is expected to improve targeting toward particular types of cells. The conjugation of RNA drug to GalNAc is the most successful example to target hepatocytes through interactions with the cell surface receptor (Nair et al., 2014, 2017). Givosiran is a GalNAc-conjugated siRNA drug (Table 2) in which the GalNAc facilitates hepatocyte uptake of givosiran to act on intracellular TTR mRNA. In other cases, aptamers can be used to facilitate targeted delivery of RNAs to specific cells, including siRNAs, miRNAs, and shRNAs (Dassie and Giangrande, 2013; Aaldering et al., 2015). Interestingly, ligand- (Davis et al., 2010) and aptamer-mediated RNA delivery (Pastor et al., 2010; Neff et al., 2011) not only increased drug efficacy but also reduced off-target effects.

As targeted delivery may facilitate the internalization of RNA drugs into targeted tissues and cells (e.g., tumor tissues and carcinoma cells), RNAs entering the systemic circulation can still be distributed into other tissues, especially liver and kidney, like small-molecule drugs. Indeed, liver and kidney are the most important organs for the elimination of xenobiotics and exogenous medications including RNA drugs (via hepatic metabolism and renal excretion, respectively), commonly showing higher levels of drug accumulation than other tissues. Interestingly, many novel RNA drugs approved by the FDA (Table 2) and currently under development are designed to act on hepatic targets. In any case, the overall efficacy and safety profiles determine whether a drug can be marketed or not, and both are attributed to the levels of drug exposure. In addition, caution must be exercised when interpreting RNA delivery/distribution/PK data obtained from studies using small-molecule dye supplemented in the RNA formulation or covalently attaching a fluorescent core to RNA as a tracer or surrogate for the convenience of analyses. On the one hand, upon release from the formulation, a small-molecule dye with its own physicochemical and PK properties that are totally different from RNA substances will be distributed or redistributed in the body in its own way, e.g., accumulated in highly proliferative tissues/cells such as tumors and carcinoma cells, whereas the RNA drugs of interest might not. On the other hand, conjugation of fluorescent core to an RNA molecule shall result in a new, different, conjugated RNA substance that undoubtedly has its own physicochemical and PK properties. Moreover, the conjugate may be metabolized in the body, and the cleaved fluorescent core will exhibit its own distribution/PK profiles. Therefore, it is vital to define the validity of such tracing approaches in related studies or directly examine the actual warhead RNAs.

3. RNA Analytical Methods. Reliable bioanalytical methods are pivotal for the quantification of drugs during discovery and development. Method validation including RNA stability, calibration range, matrix effects, sensitivity, selectivity, accuracy, and precision should be conducted in line with the regulatory guidelines on bioanalytical method validation, despite there being a lack of specific recommendations for RNAs (Kaza et al., 2019). Rather, challenges in the development of an accurate method for quantitative analysis of target RNA drug in complex biological samples seem to be overlooked. Two major strategies are commonly used for RNA analyses: hybridization-based (or biology) methods and chromatographic (or chemistry) methods (Wang and Ji, 2016). Hybridization-based assays, which may [e.g., quantitative polymerase chain reaction, (qPCR)] or may not (e.g., ELISA) involve the amplification of RNA analytes (Table 3), indirectly analyze RNAs through the use of complementary, fluorophore-labeled, or ligand-labeled oligonucleotide probes or antibodies to interact with target RNA analytes and then quantify the hybridization complexes through different readouts. On the other hand, chromatography methods aim at direct analysis of target RNAs through liquid chromatographic separation followed by detection of intrinsic UV or mass spectrometry (MS) signals (Table 3). Hybridization methods are usually highly sensitive and applicable to the analyses of all types of RNAs, whereas chromatography methods are relatively more specific and accurate in absolute quantification of sRNAs such as ASOs and siRNAs. Hybridization and chromatography may be used together in the same method, e.g., hybridization-based HPLC-fluorescence (FL) assay (Table 3).

Classic Northern blot and in situ hybridization methods are highly sensitive in detecting target RNAs through radioactivity or fluorescence detections without polymerase chain reaction–based amplification; however, these methods are semiquantitative. Conventional microarray methods and modern RNA sequencing technologies require amplification and conversion of RNAs to cDNAs,

TABLE 3
Analytical methods for oligonucleotides or RNAs

Method	Analytes	Advantages	Disadvantages
qPCR (amplification)	Unmodified oligonucleotides (chemical modifications may affect the assay)	High sensitivity, broad dynamic range (4–8 orders of magnitudes), medium to high throughput	Extensive sample preparation. Depends on primers and fluorophore. Low resolution, accuracy, and precision. Limited specificity and potential matrix effects. May not distinguish metabolites from the analyte
Hybridization-based assays or ELISA (no amplification)	Modified or unmodified oligonucleotides	High specificity (sequence dependent). Wide dynamic range. Good sensitivity. Reasonable accuracy and reproducibility	Depends on the reliability of probe, detection marker, or antibody. Possible matrix effects. May not distinguish metabolites from the analyte
LC-UV (or FL)	Modified or unmodified oligonucleotides	High specificity after separation. Good accuracy. Broad dynamic range. Low to medium throughput	Extensive sample preparation. Low sensitivity. Requires good separation (Sensitive FL detection depends on the fluorophore-labeled probe to be hybridized with the analyte)
LC-MS/MS	Small oligonucleotides (e.g., <25 nt)	High specificity (for modified RNAs), wide dynamic range, good sensitivity (for modified RNAs), medium to high throughput, good accuracy and reproducibility	Costly instrument. Limited specificity and sensitivity, particularly for unmodified RNAs

LC-MS/MS, LC tandem mass spectrometry.

which are both high-throughput approaches but offer merely relative quantification. It is also noteworthy that the efficiencies of individual RNAs can be widely variable and affected by the matrices, whereas they are generally not evaluated. For quantitative bioanalysis of RNAs, real-time qPCR (Kelnar et al., 2014) and ELISA (Humphreys et al., 2019; Thayer et al., 2019) methods (Table 3) have been commonly used. The former approach is applicable to both longer RNAs (e.g., mRNAs) and various types of sRNAs [e.g., miRNAs and siRNAs by stem-loop reverse transcription, real-time polymerase chain reaction (Chen et al., 2005)]. Nevertheless, these biological methods largely rely on the robustness of hybridization probe, amplification primers, or antibody used in the assays. Another major caveat is that these hybridization-based methods may not be able to distinguish the RNA drug from its metabolites, e.g., 1 nt deleted at the 3′ end, which the latter has been able to recognize (Ewles et al., 2014; Husser et al., 2017; Liu et al., 2019; Post et al., 2019).

After chromatographic separation, target RNA drugs can be accurately quantified by UV detection (McGinnis et al., 2012, 2013). As UV absorbance is common for nucleic acids, as well as proteins and many small-molecule compounds, the robustness of LC-UV analysis of RNA drug is dependent on efficient RNA isolation and chromatographic separation of target RNA from other components (Table 3). The use of LC tandem mass spectrometry and high-resolution accurate MS can greatly improve the specificity in quantitative analysis of ASOs and siRNAs (Watanabe et al., 2016; Liu et al., 2019) that are chemically modified and different from natural RNAs. LC-MS methods often provide good accuracy, precision/reproducibility, and wide dynamic range while offering a limited sensitivity (Table 3), dependent on the structures of RNAs and biological matrices. Compared with hybridization-based methods, LC-MS methods, especially high-resolution accurate MS, may improve the fidelity of RNA bioanalysis because they can separate and/or distinguish the metabolites from the RNA drug. With a highly demanded specificity in drug quantification, a lower sensitivity might be compromised.

4. Specificity and Safety of RNA Drugs. The specific interaction between a drug and target is critical to not only induce selective, on-target, therapeutic effects but also avoid nonspecific, off-target, adverse events. This is of particular concern because exogenous RNAs may trigger sequence-dependent and -independent immune responses. Indeed, immune-related and dose-limiting toxicities are major reasons for the failure of many RNA drugs during clinical investigations (Kleinman et al., 2008; Davis et al., 2010; DeVincenzo et al., 2010; Beg et al., 2017). The underlying targets are known as cell membrane or endosomal Toll-like receptors and cytosolic sensors (e.g., RNA-dependent protein kinase) (Karikó et al., 2005; García et al., 2006; Hornung et al., 2006; Anderson et al., 2010). The recognition of specific sequences or structures of RNA molecules by their corresponding immune receptors triggers immune events such as cytokine secretion, immune cell proliferation and survival, and adaptive immunity activation as the host's natural defense. As such, administration of pharmacological RNA agents may cause immunogenic response or cytokine release syndrome depending on the doses and structures (e.g., size, sequence, etc.) of RNA molecules (Dalpke and Helm, 2012; Tanji et al., 2015). Interestingly, although natural post-transcriptional modifications may suppress immune responses (Karikó and Weissman, 2007;

Robbins et al., 2007; Nallagatla et al., 2008; Gehrig et al., 2012), various and extensive chemical modifications can induce immunogenicity (Robbins et al., 2007; Bramsen and Kjems, 2012). Therefore, chemoengineering of RNA drugs using naturally occurring modifications (e.g., 2′-methoxyribonucleoside, pseudouridine, methyladenosine, methylcytidine, etc.) may help to reduce immunogenicity (Robbins et al., 2007; Kauffman et al., 2016). The development and use of natural RNAs made in living cells is also expected to minimize the risk of induction of immune responses (Ho and Yu, 2016; Yu et al., 2019).

While acting on targets through designed complementary base pairing, RNA agents may induce other off-target effects, particularly the interactions with unintended homologous targets. This issue is present not only in using ASOs and siRNAs for target gene silencing (Chi et al., 2003; Jackson et al., 2003; Semizarov et al., 2003) but also in utilizing gRNAs for gene editing (Frock et al., 2015; Liang et al., 2015; Filippova et al., 2019). Compared with miRNAs that may act on multiple targets, siRNAs (and ASOs) are usually postulated as specific agents in knocking down the expression of targeted genes that are rarely assessed. By contrast, genome-wide experiments showed that many transcripts with partial complementarities to siRNAs were altered in cells (Chi et al., 2003; Jackson et al., 2003; Semizarov et al., 2003). Based upon cellular miRNA-governed post-transcriptional regulation mechanism, it is hard to imagine that siRNAs do not affect other transcripts through seed-dependent complementary interactions with their 3′UTRs. Indeed, the 3′UTR matches or miRNA-like "off-target" effects have been confirmed experimentally (Birmingham et al., 2006; Jackson et al., 2006). Rather, proper design of RNAi agents can achieve relatively specific targeting and desired efficacy with minimal or manageable adverse effects, and natural miRNAs acting on multiple targets in the common pathways could be helpful for the control of particular diseases.

It is noteworthy that, like all other classes of drugs (Table 1), the selectivity and safety of RNA therapeutics are dose-dependent. Dose-limited off-target effects were commonly observed when higher or exaggerated doses of RNAi agents were used (Semizarov et al., 2003; Janas et al., 2018). Therefore, in addition to the design of optimal sequence and use of proper chemical/natural modifications to avoid or minimize immunogenicity and off-target effects, identification of the right dose to achieve therapeutic efficacy and safety is critical for the development of RNA drugs.

Since RNAs are usually delivered with particular carriers, the safety of carrier materials as well as the final formulated drug product should be critically assessed. Toxicities may come from the degradation of these materials during storage, infusion reactions of the modality, and accumulation in nontarget tissues (Vogel, 2010; Zuckerman and Davis, 2015; Szebeni et al., 2018).

The use of clinically verified safe carriers, such as LNPs, and the development of targeted delivery approaches may help to minimize the risk of toxicity caused by delivery vehicles or induced in nontarget tissues. Actually, some medications can be used proactively to protect against possible adverse effects or afterward to lessen the toxicity. This is extremely important during clinical practice because all medications may cause toxicity while also offering benefits. In terms of RNA therapies, patients may be treated with corticosteroids or antiallergy medications to manage infusion reactions to FDA-approved patisiran and investigational RNA drugs (Adams et al., 2017; Beg et al., 2017).

IV. RNAs as Therapeutic Targets for Small Molecules

A. Small Molecules Targeting Highly Structured RNAs

Although disease-related mRNAs and ncRNAs may be targeted by sRNAs such as ASOs or asRNAs, siRNAs and miRNAs described above (Fig. 1; Table 2), which are conventional small-molecule compounds, are still the preferred entities in drug discovery and development. With the understanding of mechanistic actions of many natural antibiotic drugs, including aminoglycosides, tetracyclines, macrolides, and oxazolidinones (Fig. 4), on rRNAs in the inhibition of microbial protein synthesis (Table 4) (Wilson, 2009, 2014; Lin et al., 2018), there is growing interest in discovering and developing RNA-targeted small-molecule drugs [for recent reviews, see Hermann (2016), Donlic and Hargrove (2018), Warner et al. (2018)]. The notion of developing small-molecule drugs targeting RNAs is also supported by the fact that riboswitches can be selectively bound by small-molecule ligands in the control of gene expression (McCown et al., 2017). Indeed, a number of novel classes of rRNA-targeted synthetic or semisynthetic antibiotics, including oxazolidinone (e.g., linezolid and tedizolid, etc.), ketolides (e.g., telithromycin), phenicols (e.g., chloramphenicol), and lincosamides (e.g., clindamycin), have been approved for medical use (Deak et al., 2016; Lin et al., 2018). Moreover, the first ribosome-targeted ataluren was approved in Europe for the treatment of patients with DMD with a nonsense mutation in the dystrophin gene (Haas et al., 2015), and it is undergoing phase III clinical trials in the United States (Table 4). There are also many other small molecules under clinical and preclinical development that act on existing and new RNA targets for the treatment of various human diseases, including genetic disorders, infections, and cancer (Table 4).

Similar to protein targets, macromolecule RNAs are folded into highly structured entities for their interactions with small molecules (Cruz and Westhof, 2009) or proteins (Hentze et al., 2018). Through complementary base pairings and other forms of physicochemical
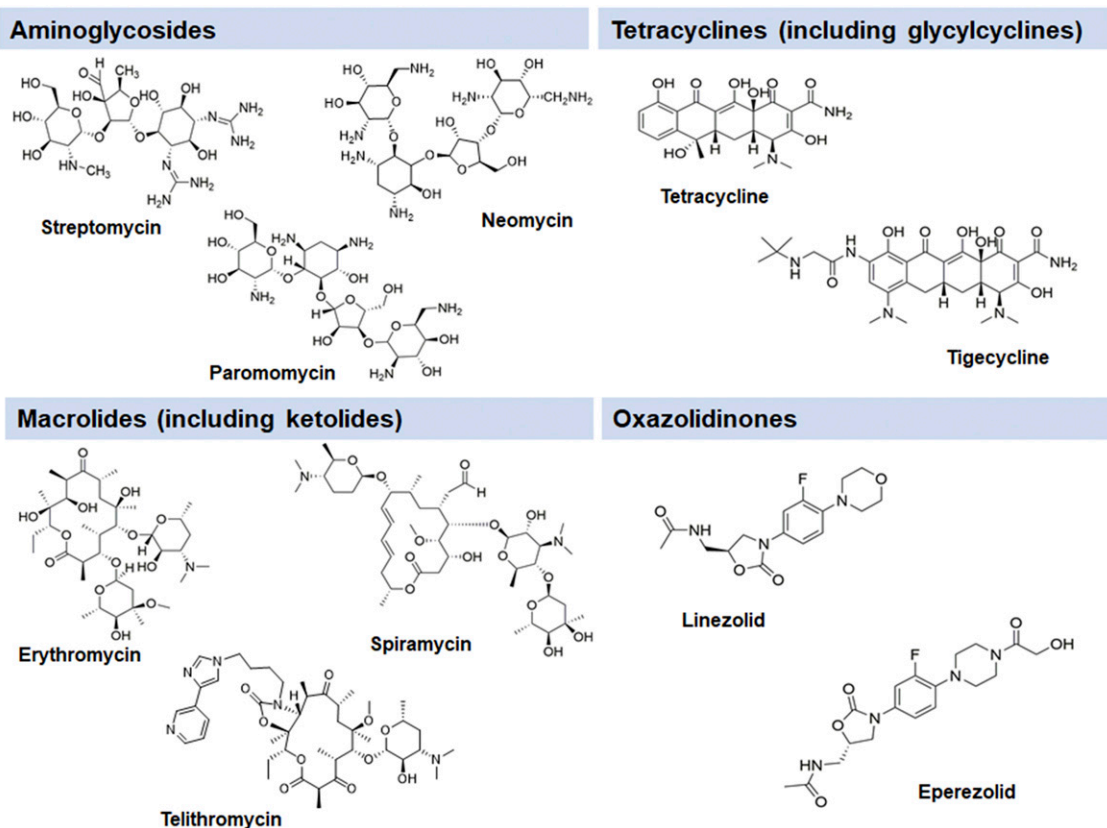
Fig. 4. Chemical structures of some small-molecule antibiotics that are known to target bacterial ribosomal RNAs to inhibit protein synthesis for the treatment of infections. Among them, aminoglycosides (e.g., streptomycin, neomycin, and paromomycin) and tetracyclines (e.g., tetracycline and tigecycline) directly act on 16S rRNAs, whereas natural and semisynthetic macrolides (e.g., erythromycin, spiramycin, and telithromycin) as well as synthetic oxazolidinones (e.g., linezolid and eperezolid) bind to 23S rRNAs to exert antimicrobial activities.

interactions, RNAs are folded into secondary (e.g., helices or stems, loops, and bulges), tertiary (e.g., junctions, pseudoknot, and motifs), and quaternary (e.g., complexes) structures (Butcher and Pyle, 2011; Jones and Ferré-D'Amaré, 2015; Schlick, 2018); thus, small-molecule compounds have the potential to directly interact with unique higher-order structures rather than the primary sequences. Specific RNA structural elements or motifs, such as bulges, loops, junctions, pseudoknots, and complexes, may be recognized and bound by particular small molecules beyond the attenuation by sRNAs with complementary sequences. Indeed, highly structured RNAs tend to contain pockets permitting specific and high-affinity binding by small molecules with particular functional groups and electrostatic surfaces, similar to those of protein targets. The interactions between small-molecule and disease-related RNA or RNA motif, therefore, could lead to the inhibition or activation of RNA functions or change of gene expression and cellular processes toward the control of disease.

Different from proteins as drug targets, which comprise a total of 22 proteinogenic amino acids, RNAs consist of only four primary nucleotide monomeric units (A, U, G, and C) as building blocks. In comparison with 22 proteinogenic amino acids, showing a combination of physicochemical properties (e.g., basic and acidic,

positively and negatively charged, hydrophilic and hydrophobic amino acids, etc.), the four monomeric nucleotides seem less variable. However, with each containing a nucleobase, ribose, and phosphate group, nucleotide monomers are much more complex than individual amino acids. As each phosphate unit carries one negatively charged residue, RNAs are highly charged macromolecules. Under low-salt conditions, many functional RNA molecules may not fold. The addition of cationic ions (e.g., magnesium ion) can induce RNA folding, whereas salt concentrations that are too high will reduce the electrostatic interactions (Lipfert et al., 2014). In addition, RNAs are generally more hydrophilic than a typical protein (McCown et al., 2017; Mustoe et al., 2018). Surrounded by an ion atmosphere under physiologic conditions, the folding and structure of electrostatic RNA, especially the sites for ligand binding, are relatively less understood, and they are very different from those found in proteins (Hermann, 2016; Morgan et al., 2017, 2018).

To identify and develop RNA-targeting small molecules, it is also necessary to understand the structural and physicochemical properties of known small-molecule ligands and recognize the principles of RNA-ligand interactions in addition to the characteristics of disease-related RNAs or RNA motifs. Indeed, most RNA-targeted

TABLE 4
Small-molecule drugs approved for clinical practice or under development that act on RNA targets

Compound	Molecular Targets and Mechanisms of Action	Clinical Application	Current Status	References
Aminoglycosides (e.g., streptomycin, neomycin, paromomycin, etc.)	Binds to 16S rRNA in the decoding region A-site on the 30S subunit to induce misreading of the genetic code and thus inhibit protein synthesis	Antibiotics to treat infections	Approved for medical use	Fourmy et al., 1996; Ogle et al., 2001; Demeshkina et al., 2012; Demirci et al., 2013
Tetracyclines (e.g., tetracycline, tigecycline, etc.)	Binds to 16S rRNA in the A-site on the 30S subunit to block tRNA binding and thus inhibit protein synthesis	Antibiotics	Approved for medical use	Brodersen et al., 2000; Anokhina et al., 2004; Schedlbauer et al., 2015
Macrolides (e.g., erythromycin, telithromycin, etc.)	Binds to 23S rRNA in the NPET of the 50S subunit to block egress of nascent polypeptide and thus inhibit protein synthesis	Antibiotics	Approved for medical use	Vannuffel and Cocito, 1996; Hansen et al., 2002; Berisio et al., 2003; Tu et al., 2005; Bulkley et al., 2010
Oxazolidinones (e.g., linezolid, tedizolid, etc.)	Binds to 23S rRNA in the A-site cleft near PTC of 50S subunit to interfere with tRNA accommodation and thus inhibit protein synthesis	Antibiotics	Approved for medical use	Ippolito et al., 2008; Wilson et al., 2008; Deak et al., 2016
Translarna (ataluren; PTC124)	Targets ribosome to promote insertion of near-cognate tRNAs at the site of the dystrophin gene toward nonsense suppression	Treatment of patients with DMD with nonsense mutation	Approved in Europe; phase III trial in the United States (NCT03179631)	Bushby et al., 2014; Ryan, 2014; Haas et al., 2015; Roy et al., 2016; McDonald et al., 2017
Risdiplam (RG7916; RO7034067)	Interacts with *SMN2* pre-mRNA and modifies RNA splicing to increase SMN protein expression levels	Treatment of patients with SMA	Phase II/III trial (NCT02913482)	Ratni et al., 2016, 2018; Poirier et al., 2018; Sturm et al., 2019
Branaplam (LMI070; NVS-SM1)	Stabilizes the spliceosome and SMN2 pre-mRNA interactions to enhance SMN2 pre-mRNA splicing and thus increase expression of full-length SMN mRNA and functional protein	Treatment of patients with SMA	Phase I/II trial (NCT02268552)	Palacino et al., 2015; Cheung et al., 2018; Dangouloff and Servais, 2019
Anthraquinones	Binds to HIV TAR element to inhibit viral replication	Treatment of HIV infection	Preclinical development	Ganser et al., 2018
Benzimidazoles (e.g., Isis-11)	Binds to HCV IRES to inhibit viral replication	Treatment of hepatitis C	Preclinical development	Seth et al., 2005; Paulsen et al., 2010; Dibrov et al., 2012
Ribocils	Binds to FMN riboswitch selectively to suppress riboswitch-targeted gene expression and thus inhibit bacterial growth	Antibiotics	Preclinical development	Howe et al., 2015, 2016; Wang et al., 2017; Rizvi et al., 2018
Targaprimir-96 and its bleomycin A5 conjugate	Binds to pri-miR-96 to inhibit Drosha-mediated processing or induce cleavage of pri-miR-96	Anticancer	Preclinical development	Velagapudi et al., 2016; Li and Disney, 2018
Targapremir-210 and its conjugate	Binds to pre-miR-210 to inhibit Dicer-mediated processing or recruit RNase L for cleavage	Anticancer	Preclinical development	Costales et al., 2017, 2019b

antibiotics (Fig. 4) derived from natural products and given to patients via intravenous administration are more complex molecules, with each containing a large number of hydrogen bond donors and acceptors, having a molecular mass over 500 Da, and being hydrophilic. This is in contrast to the majority of protein-targeted, orally bioavailable, small-molecule drugs that usually follow empirical Lipinski's rule of five (Lipinski, 2004; Ritchie and Macdonald, 2014). Therefore, caution should be exercised when adopting the rules of protein-ligand interactions for the design of RNA-targeted small molecules, although there are common principles. Moreover, some small-molecule ligands identified as highly basic and polar molecules tend to bind to RNAs with high affinity but low selectivity (Guan and Disney, 2012; Connelly et al., 2016). Thus far, a number of strategies have been developed and used to design and/or identify small molecules that can bind to RNA targets, such as binding- or phenotype-based screening (Mei et al., 1997; Sztuba-Solinska et al., 2014; Howe et al., 2015; Prado et al., 2016; Rizvi et al., 2018; Velagapudi et al., 2018; Green et al., 2019) and computational modeling and structure- or chemoinformatics-based design or virtual screening (Stelzer et al., 2011; Parkesh et al., 2012; Nguyen et al., 2015; Disney et al., 2016; Luu et al., 2016). These efforts have led to the discovery and development of chemically diverse small molecules targeting RNAs, with many exhibiting more drug-like structural and physicochemical properties (Fig. 6). In this section, representative small molecules that target various RNAs, including rRNAs, viral RNA motifs, riboswitches, pre-mRNAs, and pri or pre-miRNAs, are introduced, followed by the discussion of challenges in the identification of therapeutic RNA targets and verification of ligand binding specificity.

B. Classes of RNA Targets

1. Ribosomal RNAs. Discovery of the mechanistic actions of natural antibiotics on bacterial ribosome is a major driver for the discovery and development of RNA-targeted drugs. Bacterial ribosome (70S) catalyzing protein synthesis comprises two subunits (50S and 30S) that are assembled from three rRNA chains (16S, 23S, and 5S rRNAs) and around 55 ribosomal proteins (e.g., L16) (Steitz, 2008; Wilson, 2014; Lin et al., 2018). After the binding of 70S ribosome with the initiator tRNA and start codon of the mRNA positioned at the peptidyl-tRNA site (P-site), protein synthesis is initiated. Going through the cycle of accommodating aminoacyl-tRNA at the A-site, forming peptide bond between the A- and P-site tRNAs, translocating the peptidyl-tRNA from the A- to the P-site, and releasing the deacylated tRNAs from the exit site (E-site) that are stringently controlled by corresponding factors, a nascent polypeptide is synthesized. The termination of translation involves the hydrolysis of mature polypeptide from tRNA at the P-site, and the 70S ribosome

is recycled after the release of deacylated tRNA. The rRNA-assembled ribosome is highly structured for its interactions with individual components required for protein synthesis, in which specific clefts and pockets can be directly blocked by small molecules, leading to the inhibition of translation (Table 4).

X-ray crystallography and NMR spectroscopy studies have revealed that many aminoglycoside antibiotics, such as paromomycin and streptomycin, directly bind to 16S rRNA in the decoding region A-site on the bacterial 30S subunit to induce misreading of the genetic code and inhibit protein synthesis (Fourmy et al., 1996; Lynch et al., 2003; Demirci et al., 2013). The drug-target interactions include the involvement of crucial nucleotides, such as A1408 in 16S rRNA (Fig. 5), which when mutated (e.g., A1408G) or methylated at the N1 position (A1408m1A), confers resistance to aminoglycosides (Kondo, 2012; Kanazawa et al., 2017). The G1408 also distinguishes between prokaryotic and eukaryotic ribosomes, leading to a 25- to 50-fold lower binding affinity for paromomycin to the eukaryotic decoding site and thus providing structural explanation for the selectivity of aminoglycosides for prokaryotic ribosomes (Lynch and Puglisi, 2001). A recent study using a cryogenic electron microscopy technique has shown that the unfavorable base pairing between C1409 and A1491 in human rRNA versus perfect U1409-A1491 pairing in *Leishmania donovai* may contribute to the selective inhibition of pathogenic organisms by paromomycin instead of human ribosome (Zhang et al., 2016). Similarly, antibiotic tetracyclines (e.g., tetracycline and tigecycline) have been revealed to inhibit protein synthesis by sterically hindering tRNA binding to the A-site on the 30S subunit through direct interactions with the 16S rRNA (Brodersen et al., 2000; Anokhina et al., 2004; Schedlbauer et al., 2015). On the other hand, many natural macrolides (e.g., erythromycin, azithromycin, etc.) bind to the 23S rRNA in the nascent peptide exit tunnel (NPET) of the 50S subunit to block passage of newly synthesized polypeptides and thus interrupt translation elongation (Vannuffel and Cocito, 1996; Hansen et al., 2002; Tu et al., 2005; Bulkley et al., 2010; Dunkle et al., 2010). In addition, a lot of synthetic antibiotics designed as translation inhibitors are found to target specific sites on ribosomes (Deak et al., 2016; Lin et al., 2018). For instance, oxazolidinones such as linezolid and tedizolid directly interact with the bacterial 23S rRNA (Fig. 5) and bind in the A-site pocket near peptidyl transferase center (PTC) of the 50S subunit to exclude tRNA accommodation and thus inhibit protein synthesis (Ippolito et al., 2008; Wilson et al., 2008; Deak et al., 2016).

With the understanding of species differences in drug-ribosome interactions mentioned above, and given the importance of dysregulated protein synthesis in highly proliferative human carcinoma cells, efforts have
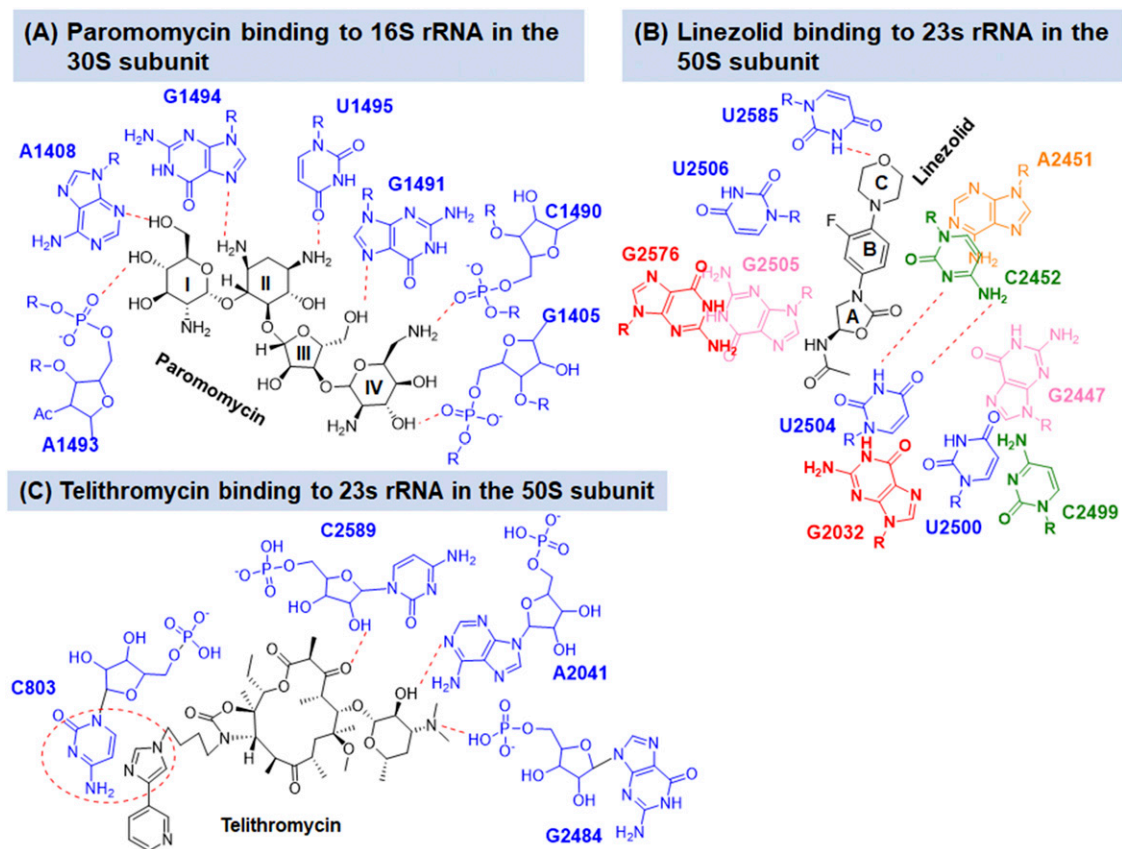
Fig. 5. Interactions of representative small-molecule antibiotics with specific nucleotide residues of individual rRNAs within ribosomal subunits. (A) Paromomycin binds to 16S rRNA in the decoding region A-site on the 30S subunit. Specifically, ring I stacks against the G1491 and interacts with A1408 through hydrogen bonding. Ring I also penetrates into the RNA helix and facilitates the flipping out of A1493. Ring II forms tight interactions with both nucleobases and the backbone of the RNA, whereas ring III just makes weak contacts. (B) Linezolid binds to 23S rRNA in the A-site cleft near PTC of the 50S subunit to interfere with tRNA accommodation. In particular, the O4 of ring-C interacts with U2585 via hydrogen bonding. Although G2576 stacks directly onto G2505, other residues clustered around U2504 form the binding pocket linezolid; among them, C2452 interacts with U2504 through the indicated hydrogen bonds. (C) Telithromycin binds to 23S rRNA in the NPET of the 50S subunit through its 3-keto group and hydrophobic interactions.

been made to elucidate the structural characteristics of human 80S ribosome and its detailed interactions with eukaryote-selective protein synthesis inhibitors (e.g., cycloheximide) (Myasnikov et al., 2016). These studies are expected to offer clues to the discovery and development of rRNA- or ribosome-targeted anticancer drugs. In addition, ribosome-targeted small molecules may be identified to interfere with the synthesis of specific protein for the treatment of genetic disorders. For example, mutations of the dystrophin gene leading to the disruption of its reading frame is a major cause of neuromuscular disorder DMD (Cirak et al., 2011), and the approval of ASO/PMO drugs eteplirsen and golodirsen (Table 2) supports the restoration of functional dystrophin protein production as a therapeutic strategy for the treatment of DMD. A small molecule, Translarna (ataluren; PTC124) (Fig. 6), has been developed to target ribosome to promote the insertion of near-cognate tRNAs at the site of the dystrophin gene and thus achieve nonsense suppression and functional protein production (Bushby et al., 2014; Ryan, 2014; Roy et al., 2016; McDonald et al., 2017). Ataluren has been approved in

Europe for the treatment of patients with DMD with a nonsense mutation in the dystrophin gene (Haas et al., 2015), and it is currently under phase III clinical trials (NCT03179631) in the United States (Table 4).

2. Viral RNA Motifs. Viral genomes consist of some highly conserved RNA elements that play pivotal roles in gene regulation and viral replication. Being highly structured, viral RNA motifs have emerged as potential targets for the development of small-molecule antiviral drugs (Hermann, 2016; Di Giorgio and Duca, 2019). The HIV transactivation response (TAR) element located within the first 59 nt of the viral genome is one of the most studied RNA elements (Stevens et al., 2006). The TAR RNA element, folded into a hairpin structure (Kulinski et al., 2003), binds to the Tat protein to form a Tat/TAR complex that stimulates transcription and HIV-1 replication (Le Grice, 2015; Connelly et al., 2016). Efforts have been made to interfere with Tat/TAR interactions to control HIV-1 infections, including the identification and development of TAR-targeted small molecules (Hermann, 2016; Di Giorgio and Duca, 2019).
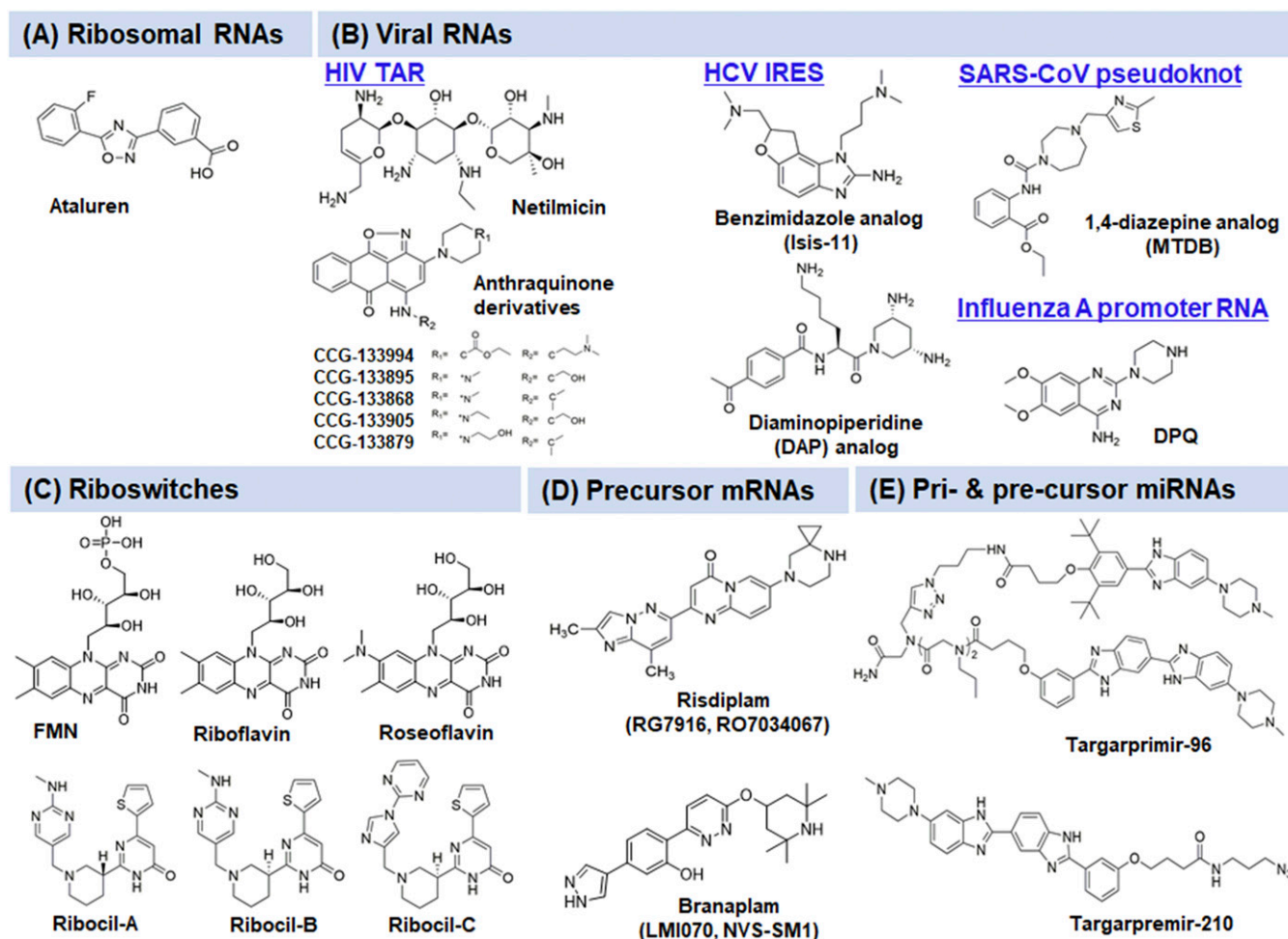
Fig. 6. Chemical structures of some small-molecule compounds under clinical or preclinical studies that act on various types of RNAs for the treatment of different diseases. (A) Ataluren targets rRNA to promote insertion of near-cognate tRNAs at the site of the dystrophin gene toward nonsense suppression for the treatment of Duchenne muscular dystrophy. (B) Netilmicin and anthraquinone derivatives targeting HIV TAR RNA motif; benzimidazole derivative Isis-11 and diaminopiperidine analog acting on HIV IRES; MTDB as an inhibitor of SAR-CoV RNA pseudoknot; and DPQ identified to attenuate influenza A promoter RNA for the control of virus infections. (C) Synthetic ribocils and natural roseoflavin mimic FMN riboswitch ligand to modulate target gene expression to exert antimicrobial activities. (D) Risdiplam and branaplam, interacting with *SMN2* pre-mRNAs to switch splicing and thus elevate the expression of functional SMN protein for the treatment of spinal muscular atrophy amenable to SMN deficiency. (E) Targarprimir-96 and targarpremir-210 that block the biogenesis of oncogenic miRNAs through direct binding to pri-miR-96 and pre-miR-210, respectively, to elicit antitumoral activities.

For example, through viral screening of TAR dynamic structures against >50,000 compounds and fluorescence-based experimental assays, one study identified and validated six small molecules that were able to bind to TAR (Kd = 55 nM to 22 μM) and inhibit its interaction with Tat (Ki = 710 nM to 169 μM) (Stelzer et al., 2011). Among them, netilmicin (Fig. 6), a semisynthetic amino-glycoside antibiotic drug, exhibited high selectivity for TAR in the presence of tRNA. The antiviral activity of netilmicin (100 μM) was further demonstrated in mammalian cell lines infected with an HIV-1 clone (Stelzer et al., 2011). Using a Tat peptide displacement assay–based high-throughput screening, the same group of investigators identified more TAR binders, including five anthraquinone derivatives (Fig. 6; Table 4) that showed selectivity relevant to tRNA (Ganser et al., 2018). Nevertheless, the efficacy and safety of TAR-targeted small molecules in animal disease models remain obscure.

Another well studied viral RNA target is the internal ribosome entry site (IRES) element located in the 5′UTR of the hepatitis C virus (HCV) viral genome (Dibrov et al., 2014; Hermann, 2016; Di Giorgio and Duca, 2019). Being a highly structured RNA motif, HCV IRES functions as an RNA-based initiation factor to directly recruit host cell ribosomes with only a subset of host cell initiation factors and thus mediates cap-independent translation initiation (Lukavsky, 2009). Through MS-based high-throughput screening against a 29-nt HCV IRES subdomain IIa, imidazoles were identified as lead binders (Seth et al., 2005). Further structure-activity relationship studies afforded a number of new analogs, e.g., Isis-11 (Fig. 6; Table 4), showing comparable binding affinity (Kd = 1.7 μM) and anti-replication activity (EC$_{50}$ = 1.5 μM) (Seth et al., 2005). Separate X-ray crystallography and NMR spectroscopy studies characterized the binding complex between HCV

IRES subdomain IIa and Isis-11 (Paulsen et al., 2010; Dibrov et al., 2012). As another example, a synthetic diaminopiperidine derivative (Fig. 6) was identified to bind with HCV IRES subdomain IIa using a fluorescence-based ligand binding assay (Carnevali et al., 2010), which likely competes with the magnesium ions, locks the IIa RNA switch in the bent conformation, and prevents ribosome release to inhibit IRES function (Dibrov et al., 2014). Although the HCV IRES arises as a promising target for the treatment of HCV infections, no small-molecule inhibitors have entered clinical trials yet.

Efforts were also made to explore other viral RNA motifs as targets for small molecules. The SARS-CoV genome consists of an atypical three-stemmed RNA pseudoknot that stimulates -1 programmed ribosomal frameshifting to initiate translation of viral proteins essential for replication (Plant et al., 2005; Su et al., 2005). Through virtual screening of about 80,000 compounds against a computationally constructed, three-dimensional SARS-CoV pseudoknot structure and experimental studies on a number of high-ranked compounds, a 1,4-diazepine analog, 2-[[4-(2-methylthiazol-4-ylmethyl)-(1,4)diazepane-1-carbonyl]amino]benzoic acid ethyl ester (MTDB) (Fig. 6), was identified to inhibit the -1 ribosomal frameshifting of SARS-CoV with an IC_{50} of 0.45 μM (Park et al., 2011). Indeed, MTDB directly interacts with SARS-CoV pseudoknot to block the formation of alternate conformers, as revealed by single-molecule force spectroscopy studies (Ritchie et al., 2014). These findings support the concept of developing small-molecule inhibitors against SARS-CoV RNA pseudoknot for the treatment of pandemic severe acute respiratory syndrome, including the ongoing SARS-CoV-2, in which the pseudoknot is well conserved.

Among most human influenza A virus variants, there is a highly conserved promoter RNA element comprising 13 nt at the 5′ end and 12 nt at the 3′ end of each viral RNA segment, which are folded together to form a partial duplex called a panhandle-like structure (Bae et al., 2001) that is recognized specifically by the RNA-dependent RNA polymerase (Pflug et al., 2014) to control transcription initiation and viral replication (Ferhadian et al., 2018). Through an NMR spectroscopy–based screening, a drug-like quinazoline compound, 6,7-dimethoxy-2-(1-piperazinyl)-4-quinazolinamine (DPQ) (Fig. 6), was found to directly bind to the influenza A virus RNA promoter (Kd around 50 μM) and exhibit antiviral activity against influenza viruses (IC_{50} = 70–300 μM) (Lee et al., 2014). Further screening and structure-activity relationship studies with cell-based anti-influenza activity assays identified a number of quinazoline derivatives that show around 10-fold higher activity against influenza A virus than the lead compound, DPQ (Bottini et al., 2015). Similar to other viral RNA-targeted small molecules described above, the discovery of quinazolines against influenza A panhandle-like promoter RNA is promising but still in its early stage.

3. Riboswitches. Riboswitches are a diverse group of natural RNA "receptors" commonly found in bacteria that directly control gene expression critical for survival (Tucker and Breaker, 2005; Hallberg et al., 2017; McCown et al., 2017). Riboswitches are usually located in the 5′UTR of particular mRNAs, and each consists of a ligand-sensing domain (or aptamer domain) and a regulatory domain (or expression platform). The specific binding of small-molecule ligand to an "aptamer" domain triggers conformational changes and stimulates the regulatory domain to govern target gene expression. Most bacterial riboswitches modulate gene expression through transcriptional or translational regulation, although some, including eukaryotic riboswitches, may provoke alternative splicing, and others can induce self-cleavage to cause mRNA degradation. In particular, various endogenous metabolite ligands, such as amino acids, vitamins, and FMN, present within cells above threshold concentrations can directly and specifically bind to the aptamer domains of riboswitches (Winkler et al., 2002b; Lee et al., 2009; Ren et al., 2015; Chauvier et al., 2017). Entrapment of a ligand can stabilize the incorporation of the switching sequence into the aptamer domain, and thus the expression platform is induced to fold into a specific structure to modulate the expression of genes controlling the biosynthesis and transport of metabolites (Serganov and Nudler, 2013; Hallberg et al., 2017).

Riboswitches have emerged as potential therapeutic targets because they are highly structured to form unique ligand binding pockets and function as gene regulatory factors, similar to the structural and functional selectivity of proteins. Many riboswitches are unique in bacteria, which upon binding to small-molecule ligands, turn off or on the transcriptional or translational expression of genes essential for pathogen survival or virulence (Zhang et al., 2004b). Therefore, small molecules may be discovered and developed to mimic or block the actions of natural ligands to interact with riboswitches with reasonable affinity and specificity for the control of infections.

Indeed, some small molecules have been identified to directly bind to targeted riboswitches, although most of them exhibit lower affinities toward the same riboswitch than natural ligand. For instance, L-lysine binds to lysine riboswitch with a Kd value around 0.36 μM, in contrast to currently identified lysine mimics, showing Kd values at 1–30 μM (Sudarsan et al., 2003; Blount et al., 2007). Likewise, thiamine pyrophosphate (TPP) binds to TPP riboswitch with high affinity (Kd = 50 nM), whereas other small-molecule binders interact with the TPP riboswitch at high concentrations (Kd $\geq$ 50 μM), except the TPP analog pyrithiamine pyrophosphate (Kd = 160 nM) (Sudarsan et al., 2005; Warner et al., 2014).

Although challenges remain in the discovery of high-affinity small molecules to target bacterial riboswitches,

recent efforts have led to the discovery of a few inhibitors with improved drug-like properties. Roseoflavin (Fig. 6), a natural analog of riboflavin and FMN, was revealed to directly bind to the aptamer domain of bacterial FMN riboswitch with a Kd value of ~100 nM to exhibit antimicrobial activity, in comparison with that of FMN (5 nM) and riboflavin (3 μM) (Lee et al., 2009; Serganov et al., 2009). Through phenotypic screening, a structurally distinct compound, ribocil (Table 4), was discovered to mimic FMN actions to suppress FMN riboswitch–mediated gene expression and inhibit bacterial growth via direct binding to the aptamer domain (Kd = 16 nM) (Howe et al., 2015). Further derivatization and structure-activity relationship studies identified ribocil-C (Fig. 6) as the most potent and highly selectively inhibitor of FMN riboswitch, showing antimicrobial activities against different bacterial strains, including medically significant Gram positive bacteria such as methicillin-resistant *Staphylococcus aureus* and *Enterococcus faecalis* (Howe et al., 2016; Wang et al., 2017). Very recently, an MS-based automated ligand detection system was established and employed for selective detection of small-molecule–ncRNA interactions, which not only verified the direct binding between ribocils and FMN riboswitch but also identified a few new binders that show greater antimicrobial activities (Rizvi et al., 2018). Although these compounds are still under preclinical investigation, these findings shall enlighten the development of a novel class of riboswitch-targeted antibiotics.

4. Precursor Messenger RNAs. Orally bioavailable small molecules (Fig. 6) have also been identified to directly interact with pre-mRNAs to modulate splicing. This strategy seems more feasible for the treatment of genetic disorders, especially those diseases with verified RNA targets. For instance, the neuromuscular disorder SMA is amenable to *SMN2* genetic variations that alter pre-mRNA splicing and thus deficiency of functional SMN protein (Aartsma-Rus, 2017; Ottesen, 2017), and targeting SMN2 pre-mRNA splicing as an effective therapeutic strategy is proven by the approval of the ASO drug nusinersen (Table 2). Indeed, a pyrimidin-4-one analog, SMN-C3, was first identified as an effective SMN2 splicing modifier through chemical screening and optimization (Naryshkin et al., 2014). Being orally bioavailable, SMN-C3 was able to increase SMN protein levels in severe SMA disease Δ7 mouse models, improve motor function, and protect the neuromuscular circuit. An SMN-C3 derivative, RG7800, was further discovered and shown to be effective in two different mouse models of SMA (Ratni et al., 2016). Although RG7800 entered clinical trials, it was terminated as a precautionary measure after the observation of retinal toxicity for chronic treatment (39 weeks) in cynomolgus monkeys (Ratni et al., 2018). Additional chemical modifications and preclinical efficacy and safety studies (Ratni et al., 2018) led to clinical development of RG7916 (risdiplam) (Fig. 6; Table 4).

As risdiplam was well tolerated among healthy volunteers and exhibited effectiveness in shifting SMN2 splicing (Sturm et al., 2019), phase II/III studies (NCT02913482) are underway to evaluate the safety and efficacy of risdiplam in patients with SMA.

Branaplam (LMI070 and NVS-SM1) (Fig. 6) represents another class of small-molecule pyridazine analogs identified to modulate SMN2 pre-mRNA splicing for the treatment of SMA caused by deficiency of SMN protein (Palacino et al., 2015; Cheung et al., 2018). Branaplam was found to bind to the pre-mRNA of SMN2 and stabilize the transient dsRNA structure formed by the SMN2 pre-mRNA and U1 small nuclear RNA protein complex, a key component of the spliceosome, which enhances SMN2 pre-mRNA splicing and increases the expression of full-length SMN mRNA and functional SMN protein (Table 4) (Palacino et al., 2015). With a favorable PK profile, branaplam was effective at improving survival of SMA Δ7 mice in a dose-dependent manner (Palacino et al., 2015; Cheung et al., 2018). Branaplam has entered a first-in-human study on the safety, tolerability, PK, pharmacological actions, and efficacy of oral branaplam in infants with type 1 SMA (NCT02268552).

5. Primary and Precursor MicroRNAs. Efforts were also made to explore small-molecule inhibitors against the biogenesis of oncogenic miRNAs in comparison with antagonism with complementary oligonucleotides as well as oncolytic miRNA replacement therapy, which was discussed before. This may be achieved through the interference with Drosha-mediated processing of pri-miRNA within nucleus or Dicer-controlled cleavage of pre-miRNA in cytoplasm because both precursors are folded into hairpin structures available for ligand binding (Fig. 7). Indeed, a computational approach has been taken to design and identify lead small molecules based on the predicted human miRNA hairpin precursor structures (Velagapudi et al., 2014). Among 27 lead compounds, a benzimidazole analog was shown to selectively inhibit the processing of pri-miR-96 into oncogenic miR-96 and thus alter miR-96 target gene expression and induce apoptosis in cancer cells. The focus on the Drosha processing site of oncogenic pri-miR-96 and optimization of benzimidazole led to the discovery of a dimeric benzimidazole and bisbenzimide compound, targaprimir-96 (Fig. 7; Table 4), which showed a favorable PK profile and was effective at releasing tumor burden in a triple-negative breast cancer xenograft mouse model (Velagapudi et al., 2016). In addition, the conjugate of targaprimir-96 with bleomycin A5 resulted in over 100-fold higher selectivity toward pri-miR-96 than DNA (Li and Disney, 2018). Very recently, another dimeric benzimidazole and bisbenzimide analog, targaprimir (TGP)-515, was identified to target pri-miR-515, leading to an induction of human epidermal growth factor receptor 2 expression levels in breast cancer cells and enhancement of the
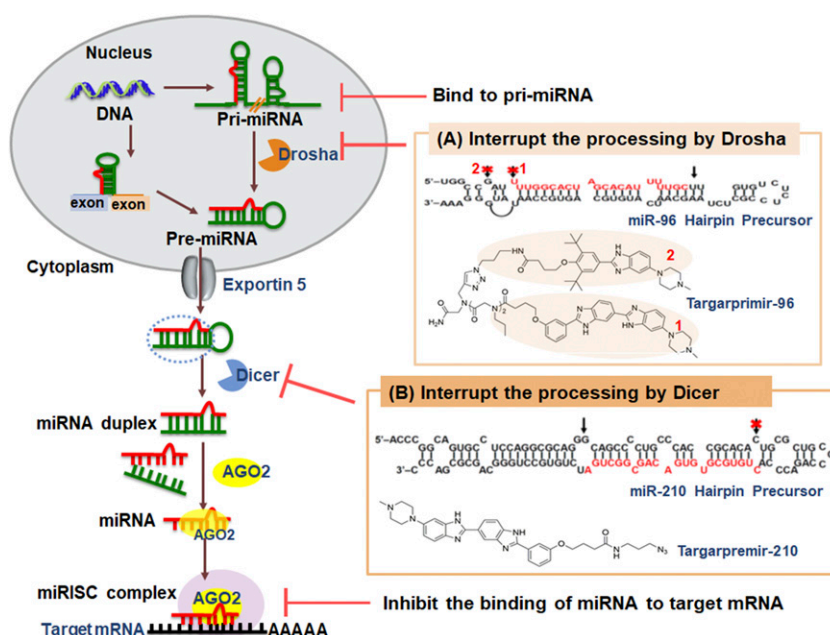
Fig. 7. Pri- and pre-miRNAs as therapeutic targets for small-molecule compounds. (A) Small molecules (e.g., the dimeric benzimidazole and bisbenzimide compound targarprimir-96) may directly bind to pri-miRNA (pri-miR-96) to inhibit Drosha-mediated processing to pre-miRNA. (B) Small molecules (e.g., the bisbenzimide analog targarpremir-210) can be identified to interfere with pre-miRNA (pre-miR-210) processing by Dicer. In addition, the binding of miRNA to target transcripts would be disrupted by small molecules, which remains to be explored.

efficacy of human epidermal growth factor receptor 2 antibody therapeutics (Costales et al., 2019a).

Likewise, a bisbenzimide (Hoechst 33342) analog called targarpremir-210, or TGP-210 (Fig. 7; Table 4), was identified to bind to pre-miR-210, leading to the inhibition of Dicer-mediated processing to mature miR-210 in human carcinoma cells and the outgrowth of xenograft tumors in mice (Costales et al., 2017). Direct binding of targarpremir-210 with pre-miR-210 was also validated by a chemical pull-down assay, and the blue fluorescent dye compound remains with a high affinity to DNA. The attachment of a nuclease (RNase L) recruitment module onto targarpremir-210 offered a conjugate, TGP-210-RL, which indeed was able to recruit RNase L onto pre-miR-210 to induce the degradation of pre-miR-210 (Costales et al., 2019b). Interestingly, this TGP-210-RL conjugate exhibited high binding selectivity to the pre-miR-210 while lacking interactions with DNA as compared with TGP-210.

A small-molecule microarray–based approach was also developed for the identification of binders of RNA motifs (Velagapudi et al., 2018). After screening against a library of 727 pharmacologically active compounds being used in the clinic or human trials, a number of DNA-intercalating agents (and topoisomerases inhibitors), such as doxorubicin, ellipticine, and mitoxantrone, as well as other entities, were revealed to bind to RNAs. Further computational and experimental studies demonstrated that mitoxantrone was able to directly bind to pre-miR-21 and subsequently inhibit Dicer-mediated biogenesis of oncogenic miR-21. Although these pharmacological agents commonly exhibit multiple

levels of mechanisms of action and druggability of these pri- and pre-miRNA inhibitors (Fig. 7; Table 4) awaits further investigations, these findings support the development of small molecules to target oncogenic ncRNAs as anticancer drugs.

6. Other RNA Targets. Some small molecules were also found to act on other types of disease-related RNAs or pathogenic RNA motifs. Many of them are for RNA repeats, such as the expanded GGGGCC repeat [$r(GGGGCC)^{exp}$, or $r(G4C2)^{exp}$, or G-quadruplex RNA], which causes frontotemporal dementia and amyotrophic lateral sclerosis (Su et al., 2014; Simone et al., 2018); the CGG repeat ($rCGG^{exp}$), which causes fragile X–associated tremor/ataxia syndrome (Disney et al., 2012; Qurashi et al., 2012; Yang et al., 2015; Green et al., 2019); and the CUG repeat $r(CUG)^{exp}$ (Parkesh et al., 2012; Luu et al., 2016; Rzuczek et al., 2017; Li et al., 2018a; Angelbello et al., 2019), which is associated with myotonic dystrophy type 1. Interestingly, there are several G-quadruplex motifs located on the 5′UTR of oncogenic KRAS transcript that could be targeted by 4,11-bis(2-aminoethylamino)anthra[2,3-b]furan-5,10-dione and 4,11-bis(2-aminoethylamino)anthra[2,3-b]thiophene-5,10-dione for the control of cancer cell proliferation and colony formation (Miglietta et al., 2017). Another polyaromatic molecule, namely RGB-1, was found to stabilize RNA G-quadruplex, leading to the inhibition of RNA translation and suppression of proto-oncogene neuroblastoma RAS viral oncogene homolog expression in breast cancer cells (Katsuda et al., 2016). In addition, a benzopyrrole analog named synucleozid was identified very recently as a binder to the structured iron-responsive element

located on the 5′UTR of α-synuclein mRNA, thus reducing α-synuclein protein levels in human cells (Zhang et al., 2020a), which may provide insight into developing small-molecule remedies for α-synuclein–caused Parkinson disease and other α-synucleinopathies.

C. Challenges in the Discovery and Development of RNA-Targeted Small-Molecule Drugs

With growing interests in developing small molecules to target RNAs for the management of human diseases (Costales et al., 2020), it should be noted that RNAs are unique macromolecules that are distinguished from proteins and are closely related to DNAs. Currently, there are a limited number of verified druggable RNA targets, and validation of a new therapeutic target requires tedious and extensive investigations. A proven therapeutic target is valid until administrative approval of respective medication. Meanwhile, studies should be conducted to critically define the specificity of a small molecule in interacting with the target RNA. Most importantly, caution should be exercised to simply adopt conventional "rules" that are applicable to protein-ligand interactions for the discovery and development of RNA-targeted small-molecule drugs. This notion is also supported by the fact that the majority of RNA-targeted antibiotics (Fig. 4; Table 4) do not follow Lipinski's rule of five, and many "RNA binders" initially designed and/or identified are known to later act on other targets (e.g., direct binding to DNAs). Therefore, structural optimization is necessary to improve the PK properties and selectivity toward a projected RNA target when developing novel efficacious and safe RNA-targeted drugs (Fig. 6).

1. Identification of Druggable RNA Targets. Two critical questions should be addressed for the identification of a druggable RNA target to control chosen disease. The first question is whether the RNA target can be accessed by small-molecule compounds. Beyond the intrinsic properties of small molecules, the accessibility of target RNA is dictated by RNA properties such as the abundance of target RNA in diseased cells, the availability of highly structured and functional sites for small-molecule binding, and so on. Indeed, rRNAs accounting for the majority of cellular RNAs (e.g., >80%) are folded to form functional binding pockets and clefts that are accessible by small molecules to intervene specifically (Fig. 5), leading to the disruption of protein synthesis and thus bacterial growth. As oncogenic miRNAs are commonly overexpressed in tumor tissues and carcinoma cells, the unique hairpin structures of miRNA precursors amenable to Drosha or Dicer processing (Fig. 7) make them promising targets for the development of small-molecule inhibitors. Rather, it is necessary to verify selective binding of small molecules with the projected RNA target among complex cellular substances, such as other forms of RNAs, as well as DNAs and large quantities of proteins.

The second question is whether interactions between small molecules and RNA targets in cells and body can lead to an effective control of disease. Prior knowledge of RNA functions related to the pathogenesis and progression of a chosen disease shall be helpful, which could have already involved extensive epidemiologic, genetic, and biochemical studies. Starting from phenotype, specific RNA targets might be pinpointed. On the other hand, the appearance or apparent management of disease with the gain or loss of RNA coding gene or function will strongly support the role of target RNA. As such, pharmacological perturbation of RNA target with small molecules becomes possible, which awaits critical and comprehensive studies using phenotypic assays and animal disease models before entering clinical investigations.

2. Specificity in Targeting RNAs. Similar to the selective actions of a drug on corresponding protein target, new RNA-targeted small-molecule drugs should bind to RNAs with reasonable specificity to elicit on-target therapeutic efficacy and avoid off-target adverse effects. This is actually a common principle for drug development, but it poses a big challenge for the discovery and development of novel RNA-targeted small-molecule modalities. As different RNA targets possess their intrinsic characteristics, the requirements for and strategies to achieve "selective" targeting may vary (Donlic and Hargrove, 2018). Although structural studies have clearly elucidated the binding of small-molecule antibiotics with rRNAs (Fig. 5; Table 2), it is inevitable that their interactions are based on the participation of essential proteins to assemble ribosomes (Wilson, 2014; Lin et al., 2018). As another example, the small-molecule splicing modifier named AMN-C not only binds to an exonic splicing enhancer ESE2 of exon 7 but also interacts with an RNA helix formed by the 5′ss intron 7 and the 5′ terminus of the U1 small nuclear RNA bound with the U1 small nuclear RNA protein to selectively alter SMN2 pre-mRNA splicing over other transcripts (Sivaramakrishnan et al., 2017).

The concern about specificity of RNA-targeted small molecules is worsened by the fact that RNAs and DNAs are both negatively charged nucleic acids and have common structural features, as well as the observation that the majority of small-molecule binders identified thus far for RNAs are either fluorescent dye derivatives (e.g., bisbenzimide or Hoechst 33342, commonly used to stain DNA), polyaromatic molecules, or highly basic or polar molecules. Many compounds themselves (e.g., doxorubicin and mitoxantrone) are well known as multifunctional DNA-intercalating agents (Wakelin, 1986). Some approaches have developed and successfully improved the selectivity toward target RNA motifs over DNA (Li and Disney, 2018; Costales et al., 2019b); however, the resulting conjugate becomes a much bigger molecule, with a molecular mass over 1500 Da, whose druggability warrants further and more extensive

investigations. In any cases, further structural modification and optimization of lead RNA binders are necessary to improve the selectivity toward RNA target for safe and effective control or eradication of particular diseases (Costales et al., 2020).

V. Conclusions and Perspectives

Unparalleled opportunities have emerged to develop the next generation of RNA-based therapeutics, including a broad range of RNA molecules as medications and disease-related RNAs as therapeutic targets for small molecules. RNA-based therapies hold the promise to greatly expand the range of therapeutic targets for safe and effective control or eradication of potentially all types of diseases. Indeed, a number of RNA drugs with novel pharmacological actions, including RNA aptamers, ASOs, and siRNAs, have been approved for medical use, and many others, such as mRNA vaccines and gRNA-mediated gene editing therapies, are undergoing active clinical trials. In addition, there is accumulating evidence supporting the mechanistic actions of existing and newly designed antibiotics on rRNAs for the control of infections, which motivates the identification of new viable RNA targets and development of novel RNA-targeted small-molecule drugs. With growing interest in and enormous efforts devoted to the development of RNA-based therapies and emerging technologies, more and more RNA-based modalities are expected to enter into clinical trials and be approved for clinical practice in the coming decades.

Different from many small-molecule and protein drugs, natural RNA molecules are negatively charged and highly susceptible to ubiquitous RNases that impede them from access to intracellular targets. Application of particular chemical modifications or derivatizations, conjugation with targeting ligands, and utilization of lipid-based carriers have been proved as effective means to improve the stability of RNAs and deliver sufficient RNA molecules into cells to exert the desired pharmacological effects, which are evident in the FDA-approved RNA drugs. However, although chemical synthesis and purification can be automated, chemoengineered RNA drugs are among the most expensive medications on the market (Simoens and Huys, 2017; Burgart et al., 2018), and the access to large quantities of synthetic RNAs required for animal studies, clinical investigations, and medical use among a large population remains a challenge. There were also concerns about inevitable effects of artificial modifications on RNA folding, activity, and safety as compared with natural RNAs produced and folded in living cells (Ho and Yu, 2016; Yu et al., 2019, 2020). Advances in RNA therapeutics will likely rely on the improvements of design and utilization of proper RNA molecules as well as delivery systems and targeting technologies to ensure efficacy and avoid or minimize off-target effects and immunogenicity.

There is also growing interest in developing orally bioavailable small-molecule inhibitors against therapeutic RNA targets beyond the use of RNA entities such as ASOs, siRNAs, and miRNAs. Because highly structured RNAs differ from proteins in many ways, including physicochemical properties and accessibility, the principles of RNA–small-molecule interactions may not necessarily follow the same rules derived from protein-targeted small-molecule drugs. By contrast, biological RNA macromolecules share common features with DNAs, although their cellular localizations can be very different. Therefore, it is necessary to verify the selectivity of small-molecule binder toward RNA target over DNAs, in addition to other types of RNAs and relevant proteins. An improved understanding of RNA structures and functions (especially those ncRNAs), identification of new pathogenic RNAs or RNA motifs as viable targets, and validation of their accessibilities for small molecules will greatly benefit future discovery and development of RNA-targeted small-molecule drugs.

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