A competitive binding assay for RNA ligand discovery

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CURIOSITY

Chapter 1

General introduction

RNA is not just a messenger

One of the most important discoveries in molecular biology is that our genetic code is stored in DNA, which is transcribed to RNA and subsequently translated to proteins. During the formulation of this central dogma[1–3], it was thought that RNA was merely a messenger molecule, which carried genetic information encoded in the DNA to the cell machinery that turned this information into proteins. However, this idea was revised with the discovery and purification of transfer RNA (tRNA)[4,5] and the distinction between what is now known as messenger RNA (mRNA) and ribosomal RNA (rRNA)[6]. Since then, many other types and functions of RNA have been found, and it has become evident that RNA is both abundant and essential in nearly all processes in the cell. RNA can be coding, meaning it carries the genetic code that will be translated into proteins (mRNA), or non-coding (ncRNA). Interestingly, ncRNAs are by far the most abundant, with only about 2% of the human transcriptome being coding RNA and 98% being ncRNA[7,8].

Non-coding RNA

ncRNAs can be divided into two main categories: 'housekeeping ncRNAs' that are involved in translation and post-transcriptional modifications, and 'regulatory ncRNAs' that are involved in regulation of gene expression (**Figure 1.1A**). Housekeeping ncRNAs are abundantly present in all cell types and are necessary for routine cell maintenance[9]. Two major housekeeping ncRNAs are tRNA and rRNA, both essential for translation. tRNA is the adaptor molecule that simultaneously decodes the mRNA sequence and carries an amino acid that can be incorporated into a growing peptide chain. rRNA is an essential component of the ribosome and is important for its structural and enzymatic function. Other housekeeping ncRNAs include small nuclear RNAs (snRNA) involved in splicing, small nucleolar RNAs (snoRNA) that assist with chemical modification of other RNAs (e.g. methylation and pseudouridylation), telomerase RNA (TERC) that catalyses telomere elongation and glycosylated RNAs (glycoRNA) that are typically displayed on the cell surface and are thought to play a role in cell signaling[10,11].

Regulatory ncRNAs exert control over gene expression at the post-transcriptional level and their expression is tissue- and cell-type specific[12]. They are generally differentiated by length (short or long) and topology (linear or circular). Short linear ncRNAs, about 20 nucleotides (nt) in length, act through degradation of target RNA and include small interfering RNAs (siRNA), microRNAs (miRNA) or PIWI-interacting RNAs (piRNA). miRNAs can also exert control through binding to mRNA sequences, thereby blocking translation.

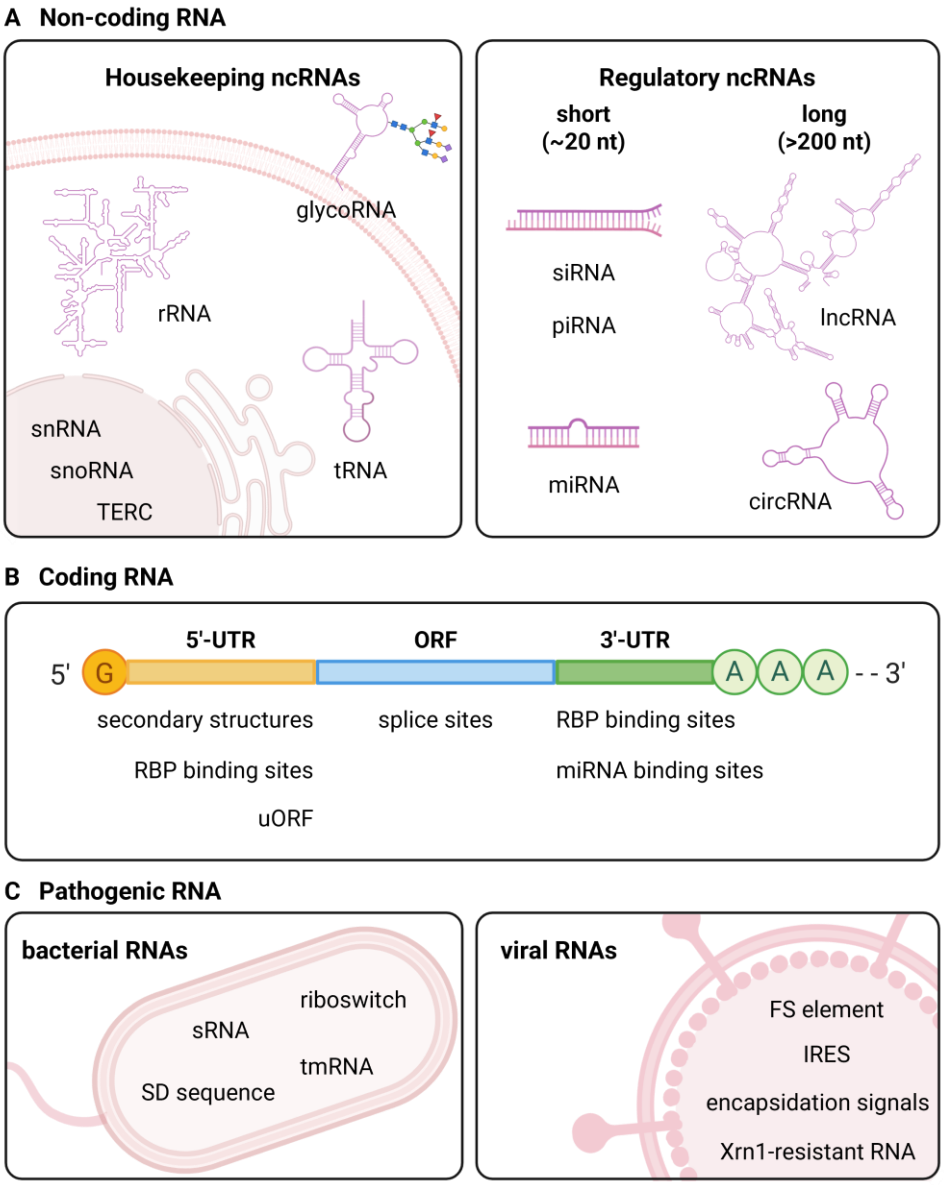

Figure 1.1. Overview of interesting human non-coding (ncRNA), coding RNA and pathogenic RNA. **A)** ncRNAs know two subcategories: housekeeping ncRNAs and regulatory ncRNAs. Housekeeping RNAs include ribosomal RNA (rRNA), transfer RNA (tRNA), glycosylated RNA (glycoRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA) and telomerase RNA (TERC). Regulatory RNAs include small interfering RNA (siRNA), PIWI-interacting RNA (piRNA), microRNA (miRNA), long non-coding RNA (lncRNA) and circular RNA (circRNA). **B)** Coding RNA, schematically visualized as an open reading frame (ORF) flanked by 5'- and 3'- untranslated regions (UTRs). Several interesting regulatory RNA elements are highlighted, including RNA-binding protein (RBP) sites and upstream ORFs (uORF). **C)** Pathogenic RNA from bacteria (left) and viruses (right), including small regulatory RNA (sRNA), transfer-messenger RNA (tmRNA), Shine-Dalgarno (SD) sequence, frameshift (FS) element and internal ribosome entry site (IRES).

Long non-coding RNAs (lncRNAs), including linear and circular RNAs (circRNA), have recently been gaining more recognition and understanding. lncRNAs make up about two-thirds of the human transcriptome[13]. Except for a minimal size of 200 nt, there are very few common structural, functional or mechanistic features that characterize all lncRNAs. Various ways of lncRNA-mediated gene regulation have been discovered, such as transcriptional or post-transcriptional regulation, epigenetic control, and regulation of nuclear architecture. Many lncRNAs act through direct interaction with DNA or RNA, and/or by formation of scaffolds that recruit or scavenge regulatory proteins (or other biomolecules, like miRNAs) to influence gene expression[9,12]. circRNAs are formed by back-splicing of linear transcripts and as a result have no termini, leading to a higher stability[14]. They are implicated in many different regulatory processes in which lncRNAs also participate[15], but can also code for proteins[16].

Coding RNA

Not only ncRNAs play important roles in gene regulation. Both coding- and untranslated regions (UTRs) of mRNAs contain *cis*-functional elements that influence gene expression (**Figure 1.1B**)[17]. These elements are mostly found in the 3′-UTR, which control for example mRNA stability, localization/or and translation, but have also been shown to mediate protein-protein interactions and post-translational modifications[18–20]. Regulation by 3′-UTRs is often achieved through binding of RNA-binding proteins (RBP) and will show different outcomes based on which RBPs are recruited and in which cellular environment the mRNA operates. Regulatory elements can be small RBP binding sites (3-8 nt)[21,22] or require the entire 3′-UTR for full functionality[23,24]. Additionally, 3′-UTRs contain miRNA binding sites that regulate degradation and/or translational repression of the mRNA[25]. The 5′-UTR is most prominently known for containing the binding sites where ribosomal subunits and initiation factors are recruited to start translation. However, it can also contain several other regulatory elements that influence translation[26], such as stable secondary RNA structures that disturb or enhance translation, RBP binding sites (e.g. iron response elements that regulate iron storage and metabolism), upstream open reading frames (uORF) that can inhibit translation of the main ORF and internal ribosome entry sites (IRES) that allow for cap-independent translation[27–29]. Coding regions contain, for example, internal splice sites that control regular and alternative splicing[30–32].

Pathogenic RNA

Pathogens also contain unique, functional RNAs that are highly conserved and essential to their lifecycle and pathogenicity (**Figure 1.1C**). With an increasing understanding of the essential role of RNA in cellular processes, more and more interesting regulatory RNAs are being discovered. In bacteria, such RNAs include riboswitches, hybrid tRNA-mRNAs (tmRNAs), small regulatory RNAs (sRNAs) and the Shine-Dalgarno (SD) sequence[33]. Riboswitches are RNA structures that are often found in the 5′-untranslated region (5′-UTR) of bacterial messenger RNA (mRNA) and can change conformation upon binding of a ligand (e.g. metabolites, co-enzymes or ions), leading to the alteration of gene expression[34–37]. Riboswitches consist of a ligand-binding aptamer domain and an expression platform that exerts genetic control, often over genes that are involved in essential cellular processes and biosynthetic pathways[38–40]. In nature, ligands are able to bind to RNA with an affinity and selectivity that is comparable to ligand-protein interactions[34], and riboswitches can have distinct small-molecule binding pockets[37]. After the experimental validation of riboswitches in 2002[41,42], more than 55 different classes of riboswitches[43], binding a wide variety of ligands[44], have been discovered and validated.

tmRNAs are hybrid tRNA-mRNA molecules that are involved in a bacterial quality control system called *trans*-translation that regulates the recycling of ribosomes and degradation of improperly translated proteins[45]. *Trans*-translation is important for bacterial fitness, development and pathogenicity. sRNAs are short RNAs (~50 to 500 nt) that regulate gene expression in bacteria by base pairing with other RNAs or associating with proteins[33]. While most sRNAs are non-essential, they are implicated in some key processes, like virulence and antibiotic resistance[46,47]. The SD sequence is the bacterial ribosome binding site located upstream of the start codon, and regulates translation through differences in sequence complementarity to the anti-SD sequence, the localization relative to the start codon and the SD sequence accessibility[48].

Viruses have limited genome size and thus require using their genomic space efficiently[49,50]. Therefore, they contain numerous RNA structures that can regulate processes like transcription, translation and replication[51]. Some notable, interesting viral RNA structures include frameshifting elements (FSEs), internal ribosome entry sites, encapsidation signals and Xrn1 resistant RNAs. FSEs are used by some viruses to regulate gene expression by inducing a process called frameshifting (FS), also known as programmed ribosomal frameshifting (PRF), which is a mechanism that regulates the relative expression of proteins from the same mRNA in different open reading frames (ORFs)[52]. FS can occur, for example, during translation when a ribosome is briefly halted

by a stable RNA structure in the FSE, which can result in the repositioning of the ribosome one nucleotide up- or downstream, continuing translation in a different ORF. Commonly, the ribosome moves one nucleotide upstream, which is called –1 FS or –1 PRF. Viral frameshifting was first observed in 1985 in the Rous Sarcoma Virus (RSV)[53], and has since been observed in many viruses, from eukaryotic viruses (such as retroviruses (HIV) and coronaviruses (SARS-CoV-1 and 2)) to plant viruses and bacteriophages[54]. Maintaining a precise level of frameshifting efficiency is known to be very important for viruses, as a disruption of the stoichiometry of the protein products, either by increased or decreased frameshifting efficiency, can significantly reduce viral replication[54–56].

Viral RNAs sometimes lack the 5′ 7-methyl guanine (m^7G) cap that is often present in eukaryotic mRNA to promote translation[57]. Instead, viruses can use internal ribosome entry sites (IRESs), usually in the 5′-UTR, that initiate translation in a cap-independent manner. Some viruses, like poliovirus[58], shut down cap-dependent translation through modification of canonical initiation factors and thereby favour viral IRES-mediated translation to give the virus a selective advantage[59–61]. Encapsidation signals, also known as packaging signals, are motifs present in viral genomic RNA(s) that bind to nucleocapsid proteins and thereby initiate the formation of virus particles. Packaging signals are therefore essential for the viral lifecycle. Both secondary structures and primary sequence of the packaging signal play a role in its function[62]. Another interesting type of RNA present in some viruses is Xrn1-resistant RNA. Xrn1 is a 5′-3′ exoribonuclease that cleaves decapped mRNA in cells, including viral RNA. By trapping Xrn1 using elaborate RNA structures in their genomes, viruses can interfere with host immune responses[63].

RNA structure

Since it is not possible to summarize all RNA functions in the limited space of a thesis, the above-described RNA motifs are highlighted to demonstrate that RNA is a fundamental part of all life on earth. To understand its role in cellular processes, it is important to understand that RNA function is inextricably linked with both its sequence and its structure. RNA is a biopolymer made from building blocks called nucleotides. Nucleotides consist of a negatively charged phosphate group, a ribose sugar and a nucleobase (guanine (G), adenine (A), cytosine (C) and uracil (U)) (**Figure 1.2A**). The phosphate groups and ribose sugars form the backbone of RNA, while the nucleobases form a specific sequence that is known as primary structure (**Figure 1.2B**).

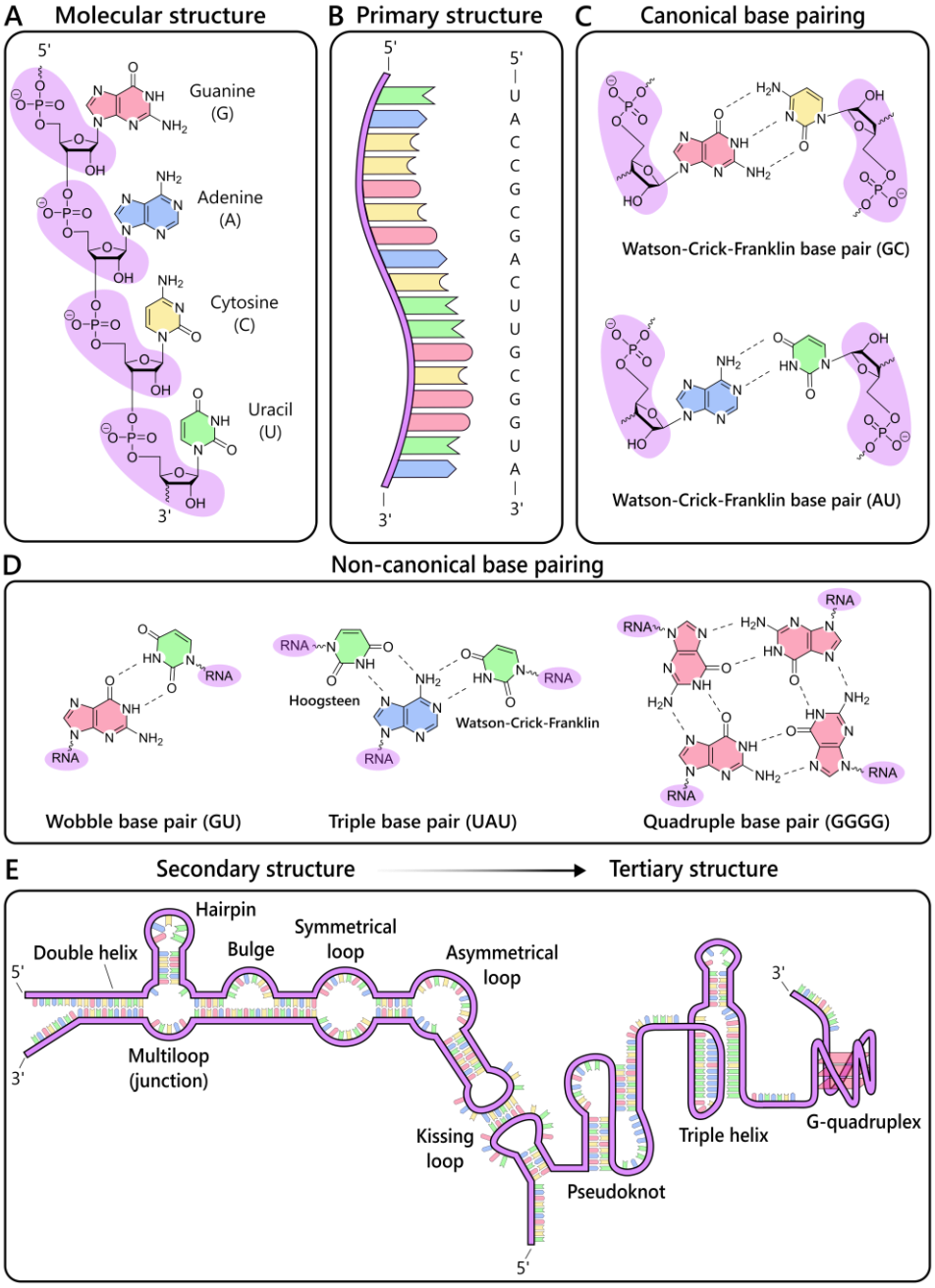

Figure 1.2. Structure of RNA. **A)** Molecular structure of RNA, with the backbone indicated in purple, and the bases indicated in red (guanine), blue (adenine), yellow (cytosine) and green (uracil). **B)** Primary structure of RNA (schematic and letter string). **C)** Molecular structure of canonical Watson-Crick-Franklin base pairing (GC and AU). **D)** Molecular structure of examples of non-canonical base pairs. **E)** Examples of common RNA secondary and tertiary structures.

In nature, RNA is rarely found in a single stranded form, as bases tend to interact with each other. The most well-known type of interaction is canonical base pairing, also known as Watson-Crick-Franklin (WCF) base pairing (**Figure 1.2C**). In WCF base pairing, a purine (G or A) and a pyrimidine (C or U) come together to form a specific hydrogen bond pattern, leading to either a GC or an AU base pair. GC base pairs have three hydrogen bonds, whereas AU base pairs have two. Alternatively, bases can form non-canonical base pairs, which is to say: any type of interaction between any two or more bases – for example, a GU Wobble base pair, a UAU triple base pair (with adenine forming one AU Hoogsteen base pair and one AU WCF base pair) and a GGGG quadruple base pair (also known as a guanine tetrad) (**Figure 1.2D**).

While there is a vast number of different types of interactions between bases, some are more stable or are formed faster than others. Generally, WCF base pairs are most stable and are formed first. A stretch of WCF base paired nucleotides is called a double helix (**Figure 1.2E**). Interruptions of double helices by unpaired or mismatched bases are called loops. These loops give rise to structural motifs such as hairpins (also called stem-loops), bulge loops, internal symmetrical- or asymmetrical loops and kissing loops. Together, these simple motifs make up the core elements of the overall RNA structure and are therefore called 'secondary structures'. Involvement of more non-canonical interactions leads to the formation of more complex structures, also called tertiary structure. This includes pseudoknots, triple helixes and G-quadruplexes, amongst others. Due to their relevance in this thesis, the structure of pseudoknots and G-quadruplexes will be explained in more detail below. Finally, quaternary structure (not shown in the figure) involves intermolecular interaction with other biomolecules, such as DNA, RNA or proteins. In cells, RNA interacts with many other biomolecules, and such interactions are very important for cellular function[64].

Pseudoknots

Pseudoknots (PKs) are complex RNA structures that received their name from having a knot-like conformation. The simplest type of pseudoknot, the hairpin (H)-type PK, forms when the nucleobases in the loop of a hairpin base pair with nucleotides adjacent to the stem of the hairpin (**Figure 1.3A**)[65]. The two resulting double-stranded helices, called stems, form one elongated, nearly-continuous helix due to coaxial stacking. The stems are connected by single-stranded loops, L1 and L3, that can form tertiary interactions, such as triple base pairs, with the nucleotides from double-stranded helices. Even amongst the relatively simple H-type PKs, there is a wide variety of possible pseudoknot conformations[66]. The final conformation of a PK depends on the length of both the stems

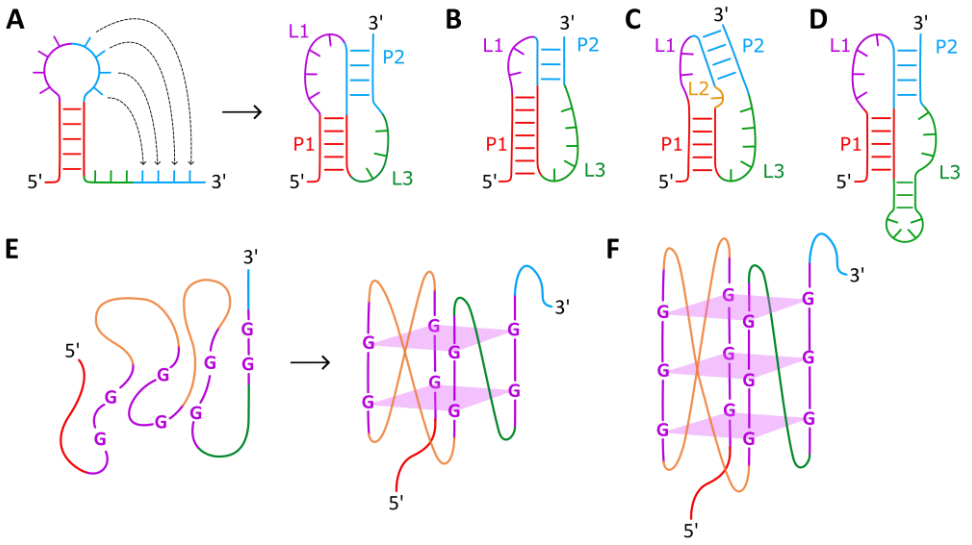

Figure 1.3. A) Folding of a hairpin (H)-type pseudoknot (PK). Indicated are stem 1 (P1), loop 1 (L1), stem 2 (P2) and loop 3 (L3). **B)** PK with different stem and loop lengths. **C)** PK with loop 2 (L2) between stacked helices and a varying relative angle between the helices. **D)** PK with a secondary structure in L3, called a HL_{out} PK. **E)** Folding of a two-tetrad G-quadruplex. Guanine tetrads are indicated in purple. **F)** A three-tetrad G-quadruplex.

and loops (**Figure 1.3B**), the presence of nucleotides (L2) between the stacked helices, the relative angle between the helices (**Figure 1.3C**), and the presence of other secondary structures in the loops, such as the HL_{out} PK that has an additional hairpin in L1 or L3 (**Figure 1.3D**)[67]. Besides the H-type PKs, there are other types with higher complexity[68]. PKs are found in many RNAs, both as an isolated motif or integrated in more complex RNA structures. The biological function of a PK is mostly dependent on its overall structure and stability, rather than its primary sequence. PKs carry out many functions, both independently (e.g. translational control and catalysis) and as central elements of various ribonucleoproteins (including ribosomes)[67,69–71]. Two notable examples of PKs in bacteria and viruses are riboswitch PKs and frameshift-stimulating PKs[52,72].

G-quadruplexes

G-quadruplexes (GQs) emerge from guanine (G)-rich sequences, in which four guanines can form a GGGG quadruple base pair, called a guanine tetrad, through Hoogsteen base pairing (**Figure 1.2D**). Tetrads can stack on top of each other through π- π stacking to form, for example, two-tetrad (**Figure 1.3E**) or three-tetrad (**Figure 1.3F**) GQs. RNA GQs are stabilized by K^+ and Na^+ ions and always exist in a parallel conformation, which means that the orientation of the RNA strands along the corners of the GQ are all in the same

direction. Because of this, the structural variety among RNA G-quadruplexes is limited. However, nucleotides in loops can provide specificity for binding of proteins or other ligands. RNA GQs can regulate transcription, translation, mRNA maturation (including splicing and polyadenylation) and transport[73–75]. Often, this is achieved through interaction with RNA-binding proteins or by interfering with the folding of functional RNA conformations[73,75,76]. GQs are involved in diseases such as neurodegenerative disease[77,78], cancer[79,80] and are present in many viral genomes[81–84].

RNA function in relation to its structure

Complexity in RNA structure arises from the great variety of interactions between bases. Interactions within an RNA strand often occur between bases that are in close proximity, but can also occur in long range, bringing together parts of an RNA that are otherwise several thousands of nucleotides apart[85]. The combination of short- and long-range interactions allows for a highly intricate RNA structure. Greater complexity is derived from modification of nucleobases. Over a hundred different natural modified nucleobases have been discovered, such as pseudouridine (Ψ), N^6-methyladenosine (m^6A), 5-methylcytidine (m^5C) and 2′-O-methylations (2′-O-Me), that influence both RNA structure and function[86].

In some cases, RNA sequence is most important to RNA function. This is best exemplified by mRNA, in which the sequence carries the genetic code that is translated to proteins. In fact, during translation all secondary and tertiary structures are unfolded by the ribosome to gain access to the sequence. However, in many cases the function of RNA is dependent on its overall structure. For those RNAs, mutations in the sequence do not necessarily lead to a loss of function, as long as the structure of the RNA remains intact[87,88]. Of course, some RNAs use both folding and specific bases to carry out their tasks, such as selective recognition of ligands by riboswitches[34,37]. It is important to note that RNAs are not static molecules, and their flexibility is paramount to their function. A single RNA strand can have multiple stable conformations and can interconvert between them due to random Brownian dynamics or through interactions with other (bio)molecules. In conclusion, despite its relatively simple molecular structure, RNA is a versatile and complex biomolecule that cleverly uses its structural features to carry out a wide range of tasks.

RNA as a drug target

Due to their essential role in protein synthesis, combined with their many other independent functions in the cell, the importance of RNAs cannot be overstated. It is therefore not surprising that dysregulation of RNA is implicated in a wide variety of human diseases[89–95]. Because of the widespread role of RNA in disease, medical biochemists have turned to targeting RNA for the development of the next generation of medicine[96]. Different types of drugs against RNA are recognized (**Table 1.1**), which can be divided into three categories: nucleic acid-, protein/peptide- and small-molecule drugs.

Targeting RNA with nucleic acids

One of the most straightforward methods to disrupt RNA function is by using complementary DNA or RNA strands, also called antisense oligonucleotides (ASOs), that base pair with an RNA target. ASOs are short nucleic acid sequences (usually between 15-25 nt in length) and can either disrupt the target RNA structure, mask its sequence, modulate its splicing or selectively induce degradation through cleavage by nucleases such as RNase H[97–99]. As of yet, targeting RNA with ASOs has been proven to be the most clinically viable therapeutic strategy[100,101], with more than 20 ASO drugs approved for diseases like Duchenne muscular dystrophy[102], spinal muscular atrophy[103] and hereditary transthyretin amyloidosis[104]. Major challenges for ASO drug development are chemical and metabolic stability, delivery[105] and off-target effects with other biomolecules[106]. Luckily, introduction of chemical modification of ASOs, such as phosphorothioates[107] or 2′-*O*-(2-methoxyethyl) (MOE) groups[100], greatly improves chemical and metabolic stability, as well as target affinity and pharmacokinetic and pharmacodynamic (PK/PD) properties. Alternatively, ASOs can be conjugated to other molecules to improve e.g. cellular uptake, delivery or biodistribution[108].

It is also possible to exploit cellular RNA silencing systems, such as small-interfering RNAs (siRNAs) and microRNAs (miRNAs), for drug development[109]. These short RNAs regulate gene expression by recruiting the RNA-induced silencing complex (RISC) to an RNA target, either to repress gene expression or to degrade the target RNA[110]. The outcome of RISC-mediated gene expression depends on the degree of sequence complementarity: targets with perfect complementarity will be degraded, whereas the presence of several mismatches will lead to translation repression. Because of their important regulatory function and programmability, siRNAs and miRNAs have potential as therapeutic

Table 1.1. Overview of RNA-targeting drugs, their highest achieved developmental stage, advantages, challenges and notable examples.

Category	Type	Development stage	Advantages	Challenges	Notable examples
Nucleic acids	Antisense oligo-nucleotides	Approved	No knowledge of target structure needed, fully programmable sequence selectivity	Stability, delivery, off-target effects, immune response	Eteplirsen (splicing modulator for Duchenne muscular dystrophy)[102] Nusinersen (splicing modulator for spinal muscular atrophy)[103] Inotersen (translation repressor for hereditary transthyretin amyloidosis)[104]
	Small interfering RNAs	Approved	No knowledge of target structure needed, fully programmable sequence selectivity	Stability, delivery, off-target effects, immune response	Patisiran (degrades transthyretin mRNA for hereditary transthyretin amyloidosis)[111] Givosiran (degrades aminolevulinate synthase 1 mRNA for acute hepatic porphyria)[112]
	MicroRNAs	Clinical trials	Potential to directly address cause of disease	Specificity, selectivity, delivery and toxicity	miR-122 inhibitor against chronic hepatitis C virus[113] miR-16 mimic for suppressing malignant pleural mesothelioma[114]
	mRNA vaccines	Approved	Scalable and cost-effective synthesis, fully programmable, safety	Stability, delivery	BNT162b2 (mRNA against SARS-CoV-2 infection)[115] mRNA-1273 (mRNA against SARS-CoV-2 infection)[116]
	DNAzymes	Clinical trials	Selectivity, easy synthesis, stability	Delivery, low *in vivo* activity	Anticancer effect against Epstein-Barr virus-associated carcinomas[117] Anti-inflammatory effect in asthma and colitis[118,119]

Category	Type	Development stage	Advantages	Challenges	Notable examples
Proteins & peptides	CRISPR/Cas13	Pre-clinical testing	No knowledge of target structure needed, fully programmable	Delivery	Splicing modulation with CRISPR Artificial Splicing Factors[120]
	RNA base editors	Clinical trials	Potential to directly address cause of disease, not permanent	Stability and delivery of guide RNA, off-target effects	RNA editing for treating alpha-1 antitrypsin deficiency[121]
	RNA-binding peptides	Approved	Selectivity, stability	Delivery, requires structural information	Viomycin (natural product cyclic peptide to block bacterial ribosomes)[122] Blocking of Tat-TAR interaction to inhibit viral replication of HIV[123]
Small molecules	Small molecules	Approved	Delivery, bioavailability, pharmacokinetics	Selectivity, requires structural information, mutational resistance	Aminoglycosides, tetracyclines and macrolides (antibiotics targeting bacterial rRNA)[124] Risdiplam (splicing modulator for spinal muscular atrophy)[125]
	Degraders	Discovery and development	Catalytic mechanism of action, potency	Molecular size, physiochemical properties	RiboSNAPs[126] PINADs[127] RIBOTACs[128,129]

modalities. siRNAs occur in cells as double stranded RNAs that are cleaved into a sense and an antisense oligonucleotide, of which the antisense strand (typically 20 to 24 nt in length) is used to guide the RISC to the RNA target and degrade it. siRNA-mediated silencing is highly efficient and sequence-specific, making it a useful tool to selectively break down disease-causing RNAs[130]. Examples of siRNA drugs approved for market use are Patisiran and Givosiran, that selectively degrade mRNA and prevent toxic accumulation of proteins involved in hereditary transthyretin amyloidosis or acute hepatic porphyria, respectively[111,112].

miRNAs are single stranded sequences of about 22 nt in length. Two types of miRNA drugs can be distinguished: miRNA mimics and miRNA inhibitors. While miRNA therapeutics have the potential to directly interfere with the root cause of disease, miRNA is promiscuous by nature, regulating multiple genes by allowing for mismatches in sequence complementarity. This, together with the fact that miRNA regulatory networks are complex, makes it difficult to achieve selectivity and avoid off-target effects[131]. Therefore, currently no miRNA therapy has been approved for market use, although several are in clinical trials[132,133]. Examples of potential miRNA therapies include an inhibitor of miR-122 against chronic hepatitis C virus[113] and a mimic of miR-16 for suppressing malignant pleural mesothelioma[114]. Important challenges for developing both miRNA and siRNA drugs are delivery, activation of immune response and toxicity. As with ASO therapies, some of these problems of can be addressed with chemical modification and conjugation to biomolecules.

A notable type of RNA drug is the mRNA vaccine. mRNA vaccines deliver mRNA to cells, which is subsequently expressed to produce the encoded protein without genetically modifying the cells. The first mRNA vaccine to be approved in the market was used to express a pathogenic antigen, the SARS-CoV-2 spike protein, in human cells to activate the immune system against a potential COVID-19 infection[115,116,134]. Delivery to cells was made possible by lipid nanoparticles (LNPs), and the mRNA code inside the LNPs could be easily adapted to include new viral strains, making it an invaluable tool for future pandemics. Unfortunately, LNPs suffered from instability and required to be stored and transported at very low temperatures. Therefore, there is still room for improvement in terms of their chemical and metabolic stability as well as mRNA delivery[135].

DNAzymes, short for DNA enzymes, are DNA structures that have catalytic activity. The first described DNAzyme was able to cleave RNA through complementary base pairing with the RNA target[136]. Subsequently, new generations of DNAzymes have been

developed that are selective for their target RNA sequence. These can easily be obtained through *in vitro* selection and are chemically stable, making them suitable for therapeutic use[137]. While DNAzymes have shown great promise against many human diseases *in vitro*[138], achieving *in vivo* activity remains challenging, due to for example issues with poor delivery and cellular uptake. However, some success has been achieved in early clinical trials with DNAzymes that downregulate a major oncogenic factor in Epstein-Barr virus associated carcinomas[117] and DNAzymes that downregulate a transcription factor involved in type 2 helper T cell-mediated inflammation[118,119,139].

Targeting RNA with proteins or peptides

In a cellular context, RNAs often interact with proteins and peptides. Using these interactions to modulate RNA function can be a therapeutic modality. Often, exploiting RNA-protein interactions requires the use of guide RNAs to gain selectivity to the RNA target. For example, CRISPR/Cas13 is a ribonucleoprotein complex part of the bacterial immune system and can selectively degrade RNA through the use of guide RNAs that recognize a specific RNA sequence. This process has been repurposed and optimised for RNA editing in eukaryotic cells, for example with modulation of alternative splicing in spinal muscular atrophy patient fibroblasts with CRISPR Artificial Splicing Factors[120]. CRISPR/Cas13 systems are fully programmable and have reduced off-target effects compared to e.g. RNA interference (RNAi) modalities like siRNA and miRNA[140]. Moreover, targeting RNA instead of DNA allows for temporary and reversible activity, therefore reducing the risk that is associated with permanent genomic changes. However, the delivery of a riboprotein complex remains troublesome, but can be achieved through nanocarriers or through delivery of vectors encoding all subunits of the CRISPR/Cas complex.

RNA base editors are enzymes that can selectively modify nucleobases to correct disease-causing mutations. For example, effector enzyme adenosine deaminases (ADARs) edit adenosine (A) to inosine (I), which is recognized by the cell machinery as guanine (G). By recruiting ADARs to sites with G-to-A mutations (which are often pathogenic mutations[141]), the wildtype genotype can be functionally restored[142,143]. Endogenous ADARs can be selectively recruited by guide RNAs complementary to the target RNA in order to site-selectively edit RNA, a strategy that is used in a clinical trial to restore mutations in the *SERPINA1* mRNA that causes alpha-1 antitrypsin deficiency[121]. Similar to other RNA-based drugs, this therapeutic modality suffers from instability, poor delivery of the guide RNA and off-target editing.

It is also possible to mimic protein-RNA interactions with peptides, in order to modulate RNA function. Such peptides can be either short fragments of an existing RNA-binding protein, newly designed synthetic peptides or natural products produced by for example bacteria. Peptides have the advantage of having a large surface area to make many specific and high affinity interactions with RNA. Their potential as an RNA-targeting therapeutic modality is demonstrated by the cyclic peptide viomycin, a market-approved antibiotic produced by *Streptomyces* bacteria that binds bacterial ribosomes and inhibits translation[122]. As of yet, no chemically designed RNA-targeting peptides have reached clinical trials, and the development of new peptides has not advanced past the discovery and development phase. Moreover, the potential of peptide therapeutics has only been researched with a handful of RNA targets. Most efforts have been directed towards finding peptides that block binding of the trans-activator of transcription (Tat) peptide to the trans-activating response element (TAR) hairpin from HIV[144–146] in hopes of disrupting viral replication. Even though the size of peptides brings forth problems with delivery and cellular uptake, chemically modifying the peptide backbone or side chains can increase stability and delivery. Especially rigidification of the peptide, by for instance head-to-tail or side chain-to-side chain cyclization, was shown to increase potency, selectivity and metabolic stability[123]. While a lot of progress has to be made, using peptides as a therapeutic modality is promising, as several cyclic peptide drugs that target proteins have been approved for market use in the past decades[147].

Targeting RNA with small molecules

The use of nucleic acids, proteins or peptides as medicine is hampered by challenges such as delivery and cellular uptake, which could be circumvented by employing small molecule-based therapies that generally have greater bioavailability, biodistribution and PK/PD properties[148]. Targeting RNA with small molecules has historically been a successful approach, as well-known small molecule antibiotics such as aminoglycosides, tetracyclines and macrolides interact with bacterial rRNA and tRNA and thereby block bacterial translation (**Figure 1.4A**)[124]. Moreover, the natural product roseoflavin[149] was discovered to exert antibacterial activity through interacting with riboswitch RNA. While these antibiotics were not specifically designed to target RNA, they serve as proof that RNA can be a drug target for small molecules. In 2020, the first small-molecule drug specifically designed to target RNA was market approved. This drug, Risdiplam (**Figure 1.4B**), is a ligand that binds to the intron-exon junction of the SMN2 transcript to correct aberrant splicing in spinal muscular atrophy (SMA)[150,125].

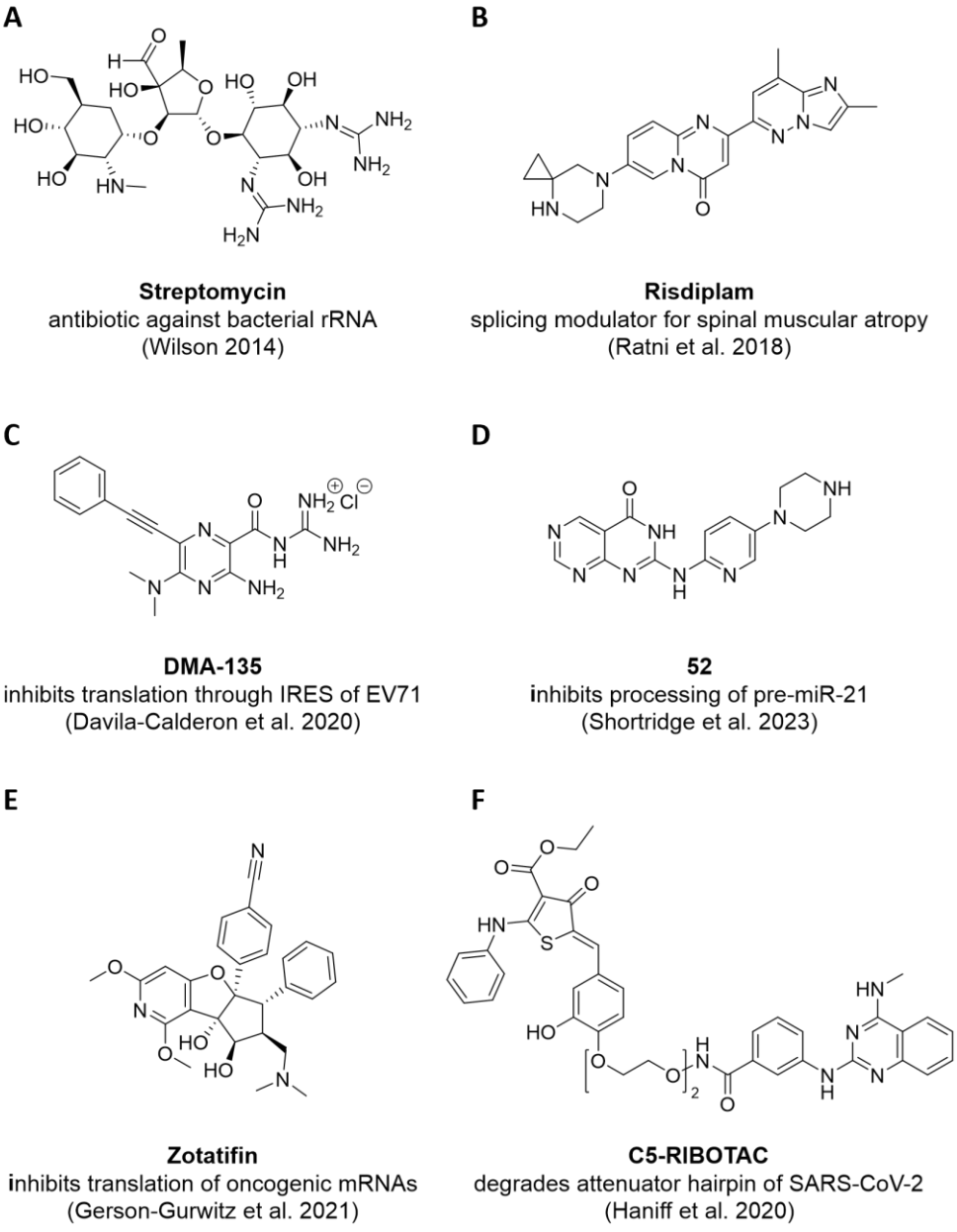

Figure 1.4. Chemical structures of small molecules targeting bacterial, viral or human RNA.

With the success of Risdiplam, there is an increased interest in developing RNA-targeting small molecules[151]. Drug discovery and development efforts are directed towards many different RNA targets, including bacterial RNAs (like the flavin mononucleotide (FMN) riboswitch[152] and T-box[153]), viral RNAs (IRES[154,155] (**Figure 1.4C**), frameshifting pseudoknots[156], TAR from HIV[157–159] and ribozymes[160,161]) and human RNAs such as lncRNAs (*Xist*[162], HOTAIR[163] and MALAT1[164]), iron response elements (IREs)[165–167] and G-quadruplexes (TERRA[168], NRAS[169] and Bcl-X[170]). Inhibiting the processing of primary miRNAs (pri-miRs) or precursor miRNAs (pre-miRs) into mature miRNAs by targeting the cleavage site of Drosha[171,172] or Dicer[173,174] (**Figure 1.4D**) has also proven to be a viable strategy for the development of anticancer drugs.

Tandem nucleotide repeats, linked to genetic disorders such as myotonic dystrophy (CUG or CCUG repeats) and Huntington's disease (CAG repeat)[175], have been targeted with small molecules that selectively bind to the repetitive loops[176–178]. Interestingly, such small molecules can be developed by rational design, connecting several loop-binding motifs with linkers of a specific length to increase selectivity and affinity. Furthermore, targeting the interface between RNA and RNA-binding proteins (RBPs) with small molecules is a good strategy, as such molecules tend to have more classical drug-like properties[151]. Tavaborole is a market approved broad-spectrum antifungal drug that inhibits $tRNA^{Leu}$ synthetase by binding to both the 3'-end of $tRNA^{Leu}$ and the enzyme[179]. Zotatifin (**Figure 1.4E**) is an anticancer drug currently in clinical trials and stabilizes the interaction between DEAD box RNA helicase eukaryotic initiation factor (eIF4A) and specific polypurine sequence motifs to inhibit the translation of certain oncogenic driver mRNAs[180,181].

Finally, a new modality for targeting RNA with small molecules is RNA degradation. RNA degraders are small molecules that can either directly cleave RNA (RiboSNAPs[126] or PINADs[127]), or recruit proteins to cleave RNA (RIBOTACs[128,129], **Figure 1.4F**). They are heterobifunctional molecules, consisting of an RNA ligand that is conjugated to an effector molecule, such as bleomycin A5 or RNase L recruiters. RNA degraders have the advantage of using a catalytic mode of action, meaning that one RNA degrader can cleave several RNA molecules, which increases their potency. Moreover, activity and selectivity can be increased by using the correct spacer between the ligand and the effector molecule[182]. However, their large size can negatively impact the physiochemical properties of RNA degraders, and the high turnover rate of RNA requires the RNA degrader to have high catalytic activity before having a physiological impact.

RNA-binding small molecules are chemically distinct from protein-binding small molecules, which is a logical consequence of the large difference in chemical structure between RNA and proteins. RNA mainly interacts with small molecules that can form electrostatic interactions with the highly negatively charged backbone or interact with nucleobases via hydrogen bonds or π-π stacking interactions. RNA-targeting small molecules generally have many heteroatom-containing aromatic rings, many hydrogen bond donors and acceptors and have a rod-like shape[183–185]. Exploring this privileged RNA-binding chemical space will help with streamlining the discovery of new ligands.

The design of new small-molecule drugs that target RNA has proven to be a significant scientific challenge. One of the main problems is achieving selective binding. Initially, it was thought to be near-impossible to selectively target RNA, as its structure is made up out of only four building blocks, which is highly limiting to its potential structural diversity. However, the discovery of riboswitches showed that interactions between RNA and ligands can be highly specific and selective[34]. On top of that, there is a distinction between binding selectivity and functional selectivity: a ligand may bind to an off-target RNA structure, but binding does not always lead to a change in the function of the off-target RNA[186]. A non-functional binding event is called 'biologically silent', which will not lead to drug side-effects. For new ligands, careful investigation of binding selectivity through transcriptome-wide binding analyses (such as ASO-Bind-Map[187], RiboSNAP[188] and Chem-CLIP-Map[189,190]), together with proteome-wide mapping and assessing functional selectivity by screening for changes in gene expression, will provide insight in off-target effects and determine whether these ligands can be considered selective.

Selectivity depends, amongst others, on uniqueness of the RNA structure, which makes knowledge of the target RNA structure crucial for drug development. While an increasing number of RNA structures are reported in the Protein Data Bank (PDB), there is still a large gap between the knowledge about structures of RNA versus proteins. Experimental structure elucidation with crystallography, cryo-electron microscopy, NMR spectroscopy and structure probing will contribute to a greater understanding of the thermodynamic and kinetic driving factors of RNA folding, which will aid in developing software to accurately model RNA structure. This, in turn, will advance drug development by identifying new targetable RNA structures and directing rational optimisation of new ligands. While the RNA-targeting field is steadily expanding, still a lot of progress is to be made.

How to find new small-molecule ligands for RNA

New RNA-binding ligands are often found with high-throughput screening (HTS) campaigns. Initial screenings were unsuccessful, as the employed small molecule libraries consisted of protein-binding molecules. With the discovery of a privileged chemical space for RNA-binding molecules, libraries were assembled that are enriched in scaffolds and moieties known to interact with RNA. Indeed, higher hit rates were found with RNA-targeting libraries[191,192], and such libraries are now screened using a variety of HTS methods[193,194], summarized below (**Figure 1.5**).

In silico screening

In silico drug discovery uses dynamic programming algorithms (such as mFold, RNAfold and RNAStructure[195–197]), together with experimental constraints for an RNA structure (found by e.g. chemical probing), to model RNA structures and subsequently dock a digital library of small molecules to find new ligands (**Figure 1.5A**). For every potential ligand, the best binding pose is predicted, scored and ranked, from which the best hits are selected. Obtained hits must then be evaluated in *in vitro* assays to determine if they indeed interact with the RNA, followed by further investigation into their affinity, selectivity and ability to disrupt RNA function. Computational screening is cheap, quick and has successfully identified several relevant RNA ligands[198]. Unfortunately, docking software requires a 3D structure of the RNA target and is therefore not applicable to every RNA target. Moreover, it is difficult to accurately take into account the flexibility of RNA in docking software, which could lead to false negatives.

Biological screening

Biological assays are usually performed in cells or cell lysates to detect ligand-RNA interactions in a natural environment. The greatest advantage of these methods is that obtained hits are already proven capable of binding to the RNA in its native conformation and interfere with its biological function. However, biological assays are often time- and resource-consuming and have a higher chance of false negatives, as such screenings do not pick up on 'biologically silent' ligands that bind to the RNA target but elicit no functional response. An often-used biological screening method is the luciferase assay (**Figure 1.5B**), in which the expression of a luciferase protein is regulated by the RNA target[199,150]. Modulation of luciferase expression through ligand binding can be easily measured in a plate reader. Unfortunately, such assays can only be used for RNA targets that can influence gene regulation and are therefore not widely applicable.

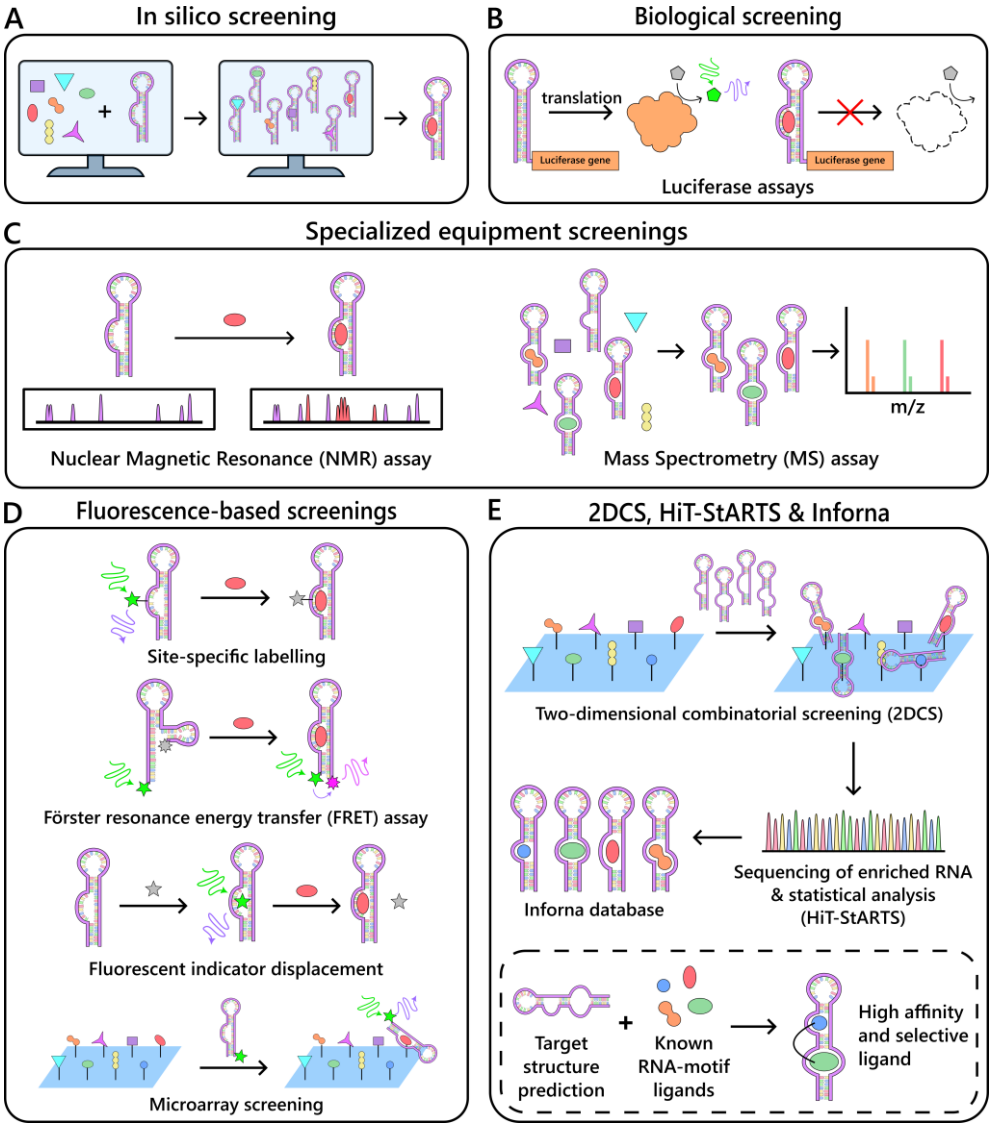

Figure 1.5. High-throughput screening methods for targeting RNA with small molecules. **A)** *In silico* screening. **B)** Biological screening with luciferase assays. **C)** Specialized equipment screenings, with for example nuclear magnetic resonance (NMR) and mass spectrometry (MS) assays. **D)** Fluorescence-based screening methods, such as site-specific fluorescent labelling, Förster resonance energy transfer (FRET), fluorescent indicator displacement and microarray screenings. **E)** Screening with the Inforna database, which was created by two-dimensional combinatorial screening (2DCS) followed by sequencing of enriched RNA & statistical analysis (HiT-StARTS). The Inforna database contains motif-specific ligands that can be combined to form high affinity and selective ligands.

Specialized equipment screenings

HTS assays that require specialized equipment, like nuclear magnetic resonance (NMR) or mass spectroscopy (MS) assays (**Figure 1.5C**), have also been used successfully for identifying RNA ligands. NMR screens make use of the fact that interactions between ligands and RNA result in a change of the chemical environment in which nuclei (such as ^{1}H, ^{13}C and ^{19}F) exist. This, in turn, leads to the appearance of new NMR peaks, peak broadening or chemical shift changes of peaks from either the ligand (ligand observed NMR)[200] or RNA bases (RNA observed NMR)[201,202]. Depending on which NMR technique is used, the screening can be performed label-free, can obtain information about binding site and stoichiometry or can be used for screening compound cocktails. Moreover, NMR is a very sensitive technique and can therefore be used for screening fragment libraries. Fragments usually have low binding affinity, but combining low-affinity fragments into a high-affinity ligand has been proven a successful strategy[203].

In MS screenings, RNA targets are incubated with potential ligands, after which ligand-RNA complexes are analysed to identify bound molecules[204]. Such screenings are very sensitive, require low amounts of sample, obtain information on binding stoichiometry and binding affinity and can screen a mixture of ligands. However, direct analysis on MS requires screening conditions that are not physiologically relevant and potentially disrupting to the RNA structure. This can be avoided by more elaborate work-up of the samples before analysis. With a technique called Automated Ligand Identification System (ALIS)[205], unbound compounds are first washed away with size exclusion chromatography. Subsequently, the bound ligands are dissociated from the target RNA and analysed with liquid chromatography-mass spectrometry (LCMS). While ALIS requires follow-up experiments to confirm target engagement, affinity and selectivity, it has proven to be a useful tool for RNA HTS campaigns[206–208]. Another HTS technique that requires specialized equipment, or rather a specialized library, is a DNA-encoded library (DEL) screening[209,210], that employs a library of DNA-tagged small-molecules to identify new ligands. Ligands are detected through sequencing of the DNA tag, making the method extremely high-throughput and very sensitive. While the above-mentioned specialized equipment techniques have been successfully applied to RNA, they require dedicated equipment and relatively elaborate data processing, which limits their widespread use.

Fluorescence-based screenings

A popular screening approach is fluorescence-based screening (**Figure 1.5D**), in which RNA targets are strategically labelled with fluorophores in order to detect small-molecule binding events or conformational changes. Site-specific labelling of RNA with fluorescent nucleobase analogues (most commonly 2-aminopurine (2-AP)) allows for the detection of small molecules that bind to a particular site in the target RNA[211,212]. Förster resonance energy transfer (FRET) assays utilize two fluorescent labels that are brought in proximity upon binding of a ligand, leading to an increase in FRET signal (or vice versa)[213–216]. Both site-specific labelling and FRET assays require either internal or terminal labelling of RNA, which can disturb the RNA conformation. This is not the case for fluorescent indicator displacement (FID) assays, in which potential ligands compete with a known fluorescent RNA ligand ('fluorescent indicator') for binding to the target RNA[217]. The fluorescent indicator changes its fluorescence properties upon binding to the RNA, and its displacement results in an increase or decrease of fluorescence signal. Indicators can be intrinsically fluorescent or can be labelled with a fluorophore. Most indicators are small molecules, although competitive displacement of fluorescently labelled peptides[159,218] has demonstrated that different types of (bio)molecules can be used, as long as it specifically interacts with the RNA target. While FID assays are very suitable for high-throughput screenings[219–221], the quality of the screening is highly dependent on selection of the correct indicator, with an appropriate binding site, affinity, and stoichiometry[217]. The above-mentioned fluorescence assays do not require highly specialized equipment, can be performed in solution and are generally fast and sensitive.

A different screening technique that uses a fluorescence read-out is microarray screening. With microarray screening, a library of potential ligands is covalently attached to a surface, to which fluorescently labelled RNA is added[222,223]. After unbound RNA is washed away, hits are identified as fluorescent spots, indicating the presence of bound RNA. Microarray screening is fast and can be performed with little material usage, although covalent attachment of the small molecules can interfere with RNA-ligand binding and requires the ligands to have a functional group for covalent linking. In an effort to avoid challenges associated with covalent binding, an immobilization technique called AbsorbArray was developed, that uses an agarose-coated surface to non-covalently adhere molecules[224]. Microarray screenings have been used for many different RNA targets and are a valuable tool for drug discovery.

2DCS, HiT-StARTs and Informa

A special case of microarray screening is two-dimensional combinatorial screening (2DCS)[225,226] (**Figure 1.5E**). In this technique, a wide variety of randomized RNA loops is screened against a large library of immobilized small molecules. The enriched RNA is analysed with a method called high-throughput structure-activity relationships through sequencing (HiT-StARTS) and from this it can be deduced which ligands have a preference for certain RNA motifs[227]. The results of these screenings were compiled in a database called Informa[228], which is now used in combination with RNA secondary structure modelling software to predict which small molecules will interact with the RNA target. This method gives a head start to the development of RNA-targeting drugs and is shown to be very successful[187,229–231]. However, Informa is currently limited to RNA secondary structures, whereas many interesting RNA targets are often tertiary structures.

While many of the above-mentioned assays are highly suitable for finding new drugs, they either require highly specialized equipment, a lot of resources or are not suitable for RNA tertiary structures. In this thesis, a fluorescence-based competitive binding antisense assay (CB ASsay) is developed to identify small molecules that target RNA tertiary structures. The assay requires minimal resources and no highly specialized equipment, making it a valuable addition to the RNA-targeting toolkit.

Targets of this thesis

The CB ASsay was developed for three RNA tertiary structures: the bacterial $PreQ_1$ riboswitch, the SARS-CoV-2 frameshifting pseudoknot and the SARS-CoV-2 G-quadruplex 'GQ-3467' (**Figure 1.6**).

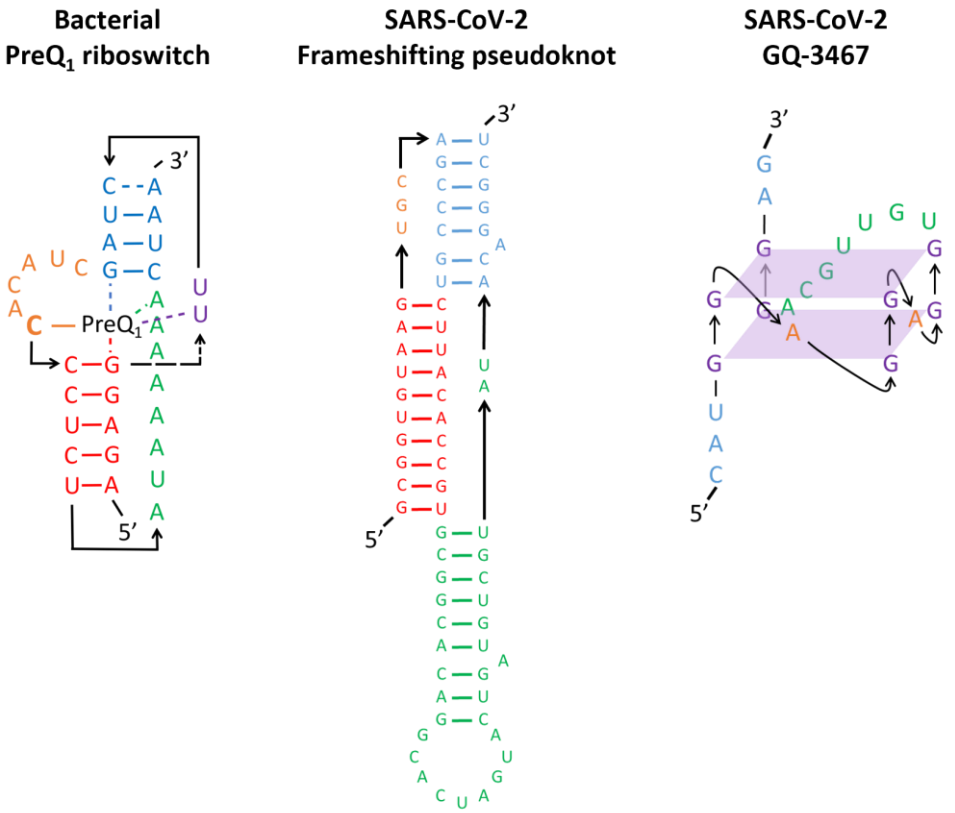

Figure 1.6. RNA targets of this thesis: the bacterial $PreQ_1$ riboswitch (left), the SARS-CoV-2 frameshifting pseudoknot (middle) and SARS-CoV-2 G-quadruplex GQ-3467 (right).

$PreQ_1$ riboswitch

The $PreQ_1$ riboswitch is a structure in the 5′-UTR of bacterial mRNA and regulates the expression of enzymes involved in the biosynthesis of a hypermodified guanine analogue called queuosine (Q). Queuosine is incorporated into the anticodon wobble position of tRNAs that share the GUN anticodon sequence ($tRNA^{Tyr}$, $tRNA^{Asp}$, $tRNA^{His}$ and $tRNA^{Asn}$)[232,233] and plays an important role in the decoding of mRNA, recognizing not only C but also U nucleotides in mRNA codons[234,235]. Interestingly, absence of Q in bacteria led to decreased viability of *Escherichia coli* and decreased virulence of *Shigella flexneri*[234,236,237]. While Q is necessary in both bacterial and eukaryotic cells, it is only

synthesized in bacteria. Because of this, the biosynthesis pathway of Q was marked as an interesting opportunity for developing antibacterial drugs.

Part of the Q biosynthesis pathway is the $PreQ_1$ riboswitch, which is known to directly regulate the expression of six proteins: four enzymes (QueCDEF) involved in the biosynthesis of $PreQ_1$ (a key precursor of Q) and two $PreQ_1$ transporter proteins (QueT and YhhQ)[238–241]. When $PreQ_1$ binds the $PreQ_1$ riboswitch, expression of these enzymes is downregulated, leading to a decreased production or uptake of $PreQ_1$ and in turn a decreased Q synthesis. Therefore, finding a synthetic ligand that can bind to the $PreQ_1$ riboswitch and ultimately decrease Q synthesis is a possible antibiotic strategy, which will be explored in Chapter 2 and 3.

SARS-CoV-2 frameshifting pseudoknot

The SARS-CoV-2 frameshifting pseudoknot (PK) is one of the most well-studied RNA structures in the SARS-CoV-2 genome. The PK is a part of the frameshifting element, which is positioned at the overlap between open reading frame 1a (ORF1a) and ORF1b and regulates the relative expression of these ORFs by facilitating a –1 programmed ribosomal frameshift that allows the ribosome to avoid the stop codon in ORF1a and continue translation in ORF1b. Translation of ORF1b is crucial for the virus, as ORF1b encodes the viral replicase machinery, such as the RNA-dependent RNA polymerase that is needed for genomic and sub-genomic RNA synthesis[242]. Moreover, maintaining the correct stoichiometry of ORF1a to ORF1b proteins is important for viral replication, as it was demonstrated that a change in frameshift efficiency results in a drastically reduced infectivity[243–246]. Since SARS-CoV-2 frameshift efficiency is dependent on the structure, dynamics and stability of the frameshifting PK, it is thought that stabilizing or disrupting the PK with a small-molecule ligand could ultimately lead to impaired viral replication. Therefore, employing the CB ASsay to find new ligands for the SARS-CoV-2 frameshifting pseudoknot will be explored in Chapter 4.

SARS-CoV-2 G-quadruplex GQ-3467

The SARS-CoV-2 genome contains several putative G-quadruplex (GQ) sequences that are considered promising antiviral drug targets[247–254], as GQs can interfere with viral translation and genome replication by blocking ribosomes or replication machinery. Among the 37 putative two-tetrad GQ sequences identified in SARS-CoV-2[247,248,255–261], the GQ sequence at position 3467 of the viral genome (GQ-3467), located in the coding sequence of non-structural protein 3 (nsp3) of ORF1a, gained particular interest. It was shown that GQ-3467 forms a stable intramolecular GQ structure, and addition of well-

known GQ-stabilizing ligands led to a higher thermal stability and decreased expression of reporter proteins both *in vitro* and *in cellulo*[248,258,262–265]. Moreover, GQ-3467 stabilizing ligands reduced viral titers in Vero E6 cells and were able to decrease viral load, lung damage and inflammation in hamster and mouse models[248,258,262–264]. While these studies demonstrate the potential of targeting GQ-3467, the tested ligands are general GQ binders and could have off-target effects. Therefore, the CB ASsay will be employed to find selective ligands for the SARS-CoV-2 GQ-3467 in Chapter 5.

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