RNA-Selective Small Molecule Ligands: The Recent Advances in Live Cell Imaging and Drug Discovery

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Abstract: RNA structures, including those formed from coding and noncoding RNAs, alternative to protein-based drug targets, could be a promising target of small molecules for drug discovery against various human diseases, particularly in anticancer, antibacterial and antivirus development. The normal cellular activity of cells is critically dependent on the function of various RNA molecules generated from DNA transcription. Moreover, many studies support that mRNA-targeting small molecules may regulate the synthesis of disease-related proteins via the non-covalent mRNA-ligand interactions that do not involve gene modification. RNA-ligand interaction is thus an attractive approach to address the challenge of "undruggable" proteins in drug discovery because the intracellular activity of these proteins is hard to be suppressed with small molecule ligands. We selectively surveyed a specific area of RNA structure-selective small molecule ligands in fluorescence live cell imaging and drug discovery because the area was currently underexplored. This state-of-the-art review thus mainly focuses on the research published within the past three years and aims to provide the most recent information on this research area; hopefully, it could be complementary to the previously reported reviews and give new insights into the future development on RNA-specific small molecule ligands for live cell imaging and drug discovery.

1. Introduction

The development of cell membrane-permeable, selective, sensitive and photostable fluorescent probes for real-time imaging and investigation of important cellular or subcellular bio-molecules and ions in live cells is of importance.[1] In recent years, significant breakthrough innovations on the function-specific organic fluorescent probes or ligands have been demonstrated in various cutting-edge applications in life sciences such as fluorescence sensing, imaging, and bio-labelling *in vitro* and/or *in vivo* in cells, tissues, and organisms.[2] At present, the applications of target-selective fluorescent ligands in chemical biology and medicinal chemistry, such as proteomic and genomic studies, disease diagnostics, pharmaceutical screening, drug delivery, protein-protein interactions, nucleic acid structure recognition, biological therapeutics, and selective *in vivo* medical or tumor imaging, are hot research areas.

Over the last three years, more than a hundred review articles focusing on bioimaging and drug discovery related development with the use of fluorescent small molecule ligands have been published, indicating a significant progress and discovery on these hot research areas. Some most recently highlighted topics with the use of target-selective fluorescent ligands include cancer cell and tissue diagnosis[2], imaging-guided drug therapy[3], disease biomarker identification[4], ferroptosis investigations[5], *in vivo* imaging[6], plasma membrane staining[7], cellular organelle imaging (targeting mitochondria[8], lysosomes[9], Golgi[10] and endoplasmic reticulum[11]), enzymatic activity[12], drug target proteins study[13], visualization of ion dynamics[14], reactive oxygen species[15], biological hydrogen sulphide[16], protein aggregation in live cells[17], bacterial labelling and infection detection[18], imaging of viscosity in living biosystems[19], imaging of microenvironments[8b] and G-quadruplex structures of DNA and RNA in live cells[20]. Among these interesting research areas, the specific area of RNA-selective small molecule ligands in fluorescence live cell imaging and drug discovery is relatively underexplored at present. This state-of-the-art review thus mainly discusses the most current studies published within the past three years and aims to provide the most recent information on this important and blooming research topic; and these up-to-date research outcomes summarized could be complementary to the previously reported review articles.

RNA, ribonucleic acid, a class of critically important biomolecules, exists in the majority of living organisms and viruses, and carries genetic information. The primary biofunction of messenger RNA (mRNA), a type of RNA in cells, is to encode various proteins, via a translation taken by ribosomes, to support different cellular activities required in live cells.[21] To investigate in depth the biofunction of RNA molecules in live cells, advanced analytical techniques are required for cellular study. Some techniques commonly utilized for gene expression analysis including polymerase chain reaction (PCR), quantitative PCR, microarrays, *in situ* hybridization methods and etc., mostly depending on the use of fixed or lysed cells, may encounter limitations to support real-time monitoring or tracking RNA targets in live cells, despite these analytical methods have been proven useful for

understanding RNA processing and regulation. To overcome these limitations and obtain the spatial and temporal information real-time on RNA dynamics including the expression, processing, transportation, localization, degradation, and storage of RNA molecules, many small molecule-based fluorescent probes selectively targeting RNA in living cells have been developed.[22] The cellular information of RNA molecules is of significance for understanding the cell function and behaviour in response to external stimuli and disease. The continued advancement of target-selective fluorescent probes for live cell imaging of RNA molecules may provide a new opportunity in a wide range of biological and medical applications against human diseases.

2. RNA Structures as the Potential Target for Small Molecules

RNA, similar to DNA, is a polymeric biomolecule that takes a broad range of biofunctions in cells, such as mRNA that typically translates the genetic information of cells into molecular machines and structures to regulating the activity of genes for various purposes.

The first study supporting RNA as a potential drug target for therapeutic intervention was the discovery of streptomycin in about 80 years ago.[23] In living systems, the genetic information is processed from DNA to mRNA (the coding RNA) and followed to protein. Moreover, the noncoding RNA molecules generated from DNA transcription serve as the guiding molecule and may participate in the assembly of proteins specified by mRNA molecules to reach their specific location and then to exert their pre-set biological functions in cells. Ribosomal RNA (rRNA) builds up the enzymatic framework of the ribosome that is the core machine to produce proteins by following the instructions or codes given in a mRNA sequence with the use of transfer RNA (tRNA) molecules.

Gene expression in cells is strictly controlled by RNA molecules. The regulation of protein synthesis from different mRNA is a core and fundamental process for living organisms to maintain their cellular structure, differentiation and physiology. In addition, RNA molecules may fold into unique secondary and tertiary structures that drive diverse functional activities in cells.[24] Some examples of human RNA structures[21] regulating key biological functions are shown in **Figure 1A**. The normal function of cells is critically dependent on the accurate expression of various mRNA and noncoding RNA molecules and thus mutations may disrupt the normal cellular function and cause various human diseases such as cancer.[25] Despite the coding RNA molecules are just the intermediate between DNA and protein, they are considered as critically important targets for drug discovery because they regulate many key biological processes in cells. For example, in the human genome, there are about 75% genes that may provide approximately 50,000 potential drug targets transcribed into RNA molecules, and about 1.5% genes that could possibly provide about 20,000 potential drug targets are translated into proteins.[21, 26] The typical RNA targets for drug discovery including mRNA, pre-mRNA, tRNA, rRNA, miRNA and long noncoding RNA have been intensively studied over the last decade.[21]

In addition, a great number of guanine(G)-rich sequences of nucleic acids, including DNA and RNA, capable of forming G-quadruplex (G4) structures that are three-dimensional and noncanonical secondary structures, have been reported.[27] The formation of these noncanonical secondary structures of nucleic acid *in vitro* has been proved with high-resolution *X*-ray crystal structures while their existing *in vivo* remains controversial currently.[27b, 28] Some models of RNA G4-structure are shown in **Figure 1B**.[29] Recent studies also support that the formation of G4-structures in the transcriptome could be a dynamic process,[30] indicating that the *in vivo* existing of G4-structures may be transient and regulated with enzymes in cells. Nonetheless, the dynamic nature of the G4 folding-unfolding process may support the biological significance of these special structures in the regulation of RNA metabolism.[29, 31] Furthermore, RNA G4-mediated functions may ultimately be connected to mechanisms underlying disease pathologies and may also provide opportunities for the development of new RNA-targeted therapeutics.[32]

To interrupt the cellular function of disease-relevant RNA structures, various RNA binding small molecule ligands, such as translation inhibitors, splicing modulators, protein-binding inhibitors, competitive inhibitors and RNA-targeted degradation agents, have been developed.[21, 25b, 33] Despite RNA is currently considered as the challenging drug target, the regulation of RNA functions through non-covalent structure-ligand interactions has been considered as a promising approach for drug discovery against human diseases. The understanding of interactions between RNA and lingands is thus very important. The RNA–ligand interaction properties obtained could provide critical information on the vital processes in molecular biology because the targeted RNA may also interact with various essential biological molecules such as DNAs, other RNAs and proteins in live cells. Several review articles have recently discussed RNA–ligand interactions on the fundamental recognition principles and noncovalent interactions, and the functional role of RNA tertiary structures and specific binding modes.[34] Despite the structure of RNA in terms of tertiary folding exhibits a high level of diversity, there are a number of useful methodologies developed for molecular recognition and the investigation of the RNA-ligand complex formed *in vitro* and *in cellulo*.

Generally, there are two common experimental based apporaches for RNA–ligand interaction study: (i) Biophysical methods mainly including fluorescence-based methods, isothermal titration calorimetry, and surface plasmon resonance are commonly used to study RNA–lingad interactions.[35] (ii) The structural approach based on solid state methods includes *X*-ray crystallography and cryo-EM/cryo-TEM, and solution-state methods such as NMR spectroscopy and chemical probing study.[36]

Despite these experimental based techniques are the powerful tools for RNA–lingand interaction investigations, they do have their own limitations such as a high resolution *X*-ray structural of a RNA–ligand complex is not readily obtainable. The prediction of three-dimensional RNA conformations and structures also provides great assistance in the design of RNA molecules with specific structural properties for RNA-based therapeutics. In recent years, computer-aided design of RNA-targeted small molecules shows a growing need in drug discovery.[37] The development of computational and artificial intelligence based tools may predict and simulate RNA–ligand interaction and the bindng mode of the complex, which could provide meaningful information not observable with experimental methods.

3. Fluorescent Imaging of Cellular RNAs with Small-molecule Ligands

RNA molecules are the key players in regulating various cellular functions in live organisms. The transformation of genetic information from DNA into mRNA and functional proteins for execute a specific biofunction is a complicated and multi-stage process. The monitoring of RNA molecules and their associated activity in live cells is thus significant. The in-depth understanding of RNA functions and metabolisms is useful for chemical biology study and drug discovery.[21-22] It is well-known that the life-cycle of RNA molecules is initiated from transcription and followed splicing, transport, localization, translation and degradation.[38] However, RNA molecules are non-emissive biomolecules and thus they are hard to trace and monitor in cells. The mechanism and dynamics involved in each step of RNA processing are therefore difficult to monitor and understand clearly. Fluorescent imaging techniques for visualizing RNA molecules in live cells could provide valuable and convenient tools for investigations.[20b, 22a, 22c] Currently, there are several advanced cellular imaging methodologies with the use of fluorescent microscopy developed for investigating the spatial and temporal resolution in molecular and cellular biology of RNA molecules. Depending on the need of the study, RNA molecules can be selectively visualized in both fixed and live cells. A number of latest review articles have concluded the recent research progress on RNA biology using fluorescence imaging as the powerful tool.[22] To avoid redundant, the reviewed RNA imaging systems are not going to be discussed in the present study.

A number of RNA-specific imaging methods, such as fluorescence *in situ* hybridization (fliFISH)[39], single-molecule FISH (smFISH)[40], rolling-circle amplification (RCA)-FISH[41] and click-amplifying FISH (clampFISH)[42], hybridization chain reaction (HCR)-FISH[43], signal amplification by exchange reaction (SABER)-FISH[44], multiplexed error-robust fluorescence *in situ* hybridization (MERFISH)[45] and etc., have been developed for RNA investigations. In addition, sub-cellular multiplexed RNA imaging in fix cells can be achieved by using combinatorial FISH and *in situ* sequencing.[22a] Furthermore, to image RNA molecules in live cells, fluorescently labelled RNA, RNA stem-loop, and fluorogenic RNA are useful methods for the study of exogenous RNA molecules. However, these imaging techniques are not able to visualize endogenous and non-genetically modified mRNA molecules. Alternatively, cell-membrane and/or nuclear-membrane permeable small molecule probes/ligands and the genetically encoded probes could be utilized for imaging endogenous mRNA targets in live cells.

Over the past few years, from 2020 to 2022, more than ten review articles have been published and highlighted the development of advanced molecular fluorescent probes for intracellular RNA imaging,[20b, 21-22, 22c, 46] which may evidence the significance and attractiveness of RNA biology and RNA-based therapeutics utilizing the fluorescence turn-on molecules as a powerful tool. One of the popular fluorescence techniques is the use of fluorogenic RNA aptamer-dye pairs, a genetically encodable biosensor, for live cell imaging study. These fluorogenic small molecules such as Broccoli, Spinach, Corn, Mango and HBC (Peppers) and their applications in RNA investigations have been reviewed recently.[22c, 46a, 47] The tagging method for a fluorogenic RNA aptamer-dye pair requires the modification of an RNA of interest. The genetically encoded aptamers can also be transcribed by cells on their own for intracellular investigations (**Figure 2 A-B**). The availability of a suitable binding domain is a general limitation for the development of genetically encodable biosensors. Nonetheless, imaging of a single mRNA with fluorogenic RNA aptamers and small molecule fluorophores is achievable currently.[46a] In addition, the endogenous RNA of interest can be detected and studied with hybridization-based probes (oligonucleotide-based probes or termed molecular beacon),[48] which are single-stranded short hairpin probes or molecules with an internally quenched fluorophore as shown in

Figure 2C. The oligonucleotide-based probes specifically hybridize with endogenous RNA of interest and then the fluorescence is regenerated.

Apart from genetically encoded aptamers and hybridization-based probes, RNA-selective fluorescent binding probes based on small molecule ligands have been developed for imaging of RNA in live cells.[49] These molecular probes usually exhibit low or no background fluorescence in the unbound state in solution and show significantly enhanced fluorescence upon binding to the RNA target through non-covalent interactions. For intracellular applications to image endogenous RNA targets, fluorescent probes have to be cell-membrane and/or nuclear membrane permeable. Moreover, the molecular probes that can be excited in visible light region or at longer wavelength and emit in deep red or near infrared (NIR) region are more preferred for *in cellulo* and *in vivo* study[50] because these probes could reduce largely or avoid photobleaching and autofluorescence. In addition, the excitation light source such as NIR may has better tissue penetration. In fluorescent ligand design targeting biomolecules such as RNA in live cells, to extend the π-conjugation system of a fluorophore may generally shift its absorption maxima toward longer wavelength (red shift); however, it may also increase the molecular size and hydrophobicity of the probe and that causes poor water solubility and bioavailability. In general, fluorescent probes with poor water solubility and photostability may not be favourable for intracellular and *in vivo* study. The small-sized and red (or NIR) fluorescent ligand is thus more preferable. Furthermore, the selectivity, sensitivity and stability of a fluorescent ligand toward the target of interest are the critical factor of consideration for *in cellulo* and/or *in vivo* applications.

Currently, the reported small molecule-based fluorescent probes targeting RNA and DNA secondary structures are mostly with emission in the visible light region ranged from 450–650 nm while the probe emitting in the range of 660–750 nm is not common.[20a, 20b, 22a, 22c] This may imply that the development of red or NIR fluorescent probes for RNA imaging is challenging and more efforts may be required for further advancement in the future. With respect to literature, the number of small molecule-based fluorescent probes targeting RNA reported is still limited at present. Most RNA-selective fluorescent probes such as those shown in **Figure 3** and their features in RNA sensing and imaging in live cells have been reviewed recently.[22d, 51] In the following, only those small molecule ligands not included in the reviews published previously are selected for a detailed discussion on their photophysical property and fluorescence sensing performance targeting RNA molecules.

Two small molecule-based fluorescent probes, **Styryl-TO** and **PI2**,[52] were found highly selective targeting ribosomal RNA (rRNA) *in vitro* and in live human cancer cells. The ligands share a common cationic scaffold, 1-methylquinolinium, which is substituted with different free rotatable substituents, including benzothiazole and indole groups, at both 2- and 4-position. **Styryl-TO** is designed by integrating a thiazole orange (a non-selective nucleic acid staining agent) and a *p*-(methylthio)styryl scaffold. The ligand shows better nucleolus RNA imaging performance in live cells than a commercial RNA dye, SYTO RNASelect™. The ligand in buffer solution shows almost no background fluorescence and generates strong interaction signal upon binding to RNA substrates (λ_{ex} = 520 nm; λ_{em} = 531 nm) and the equilibrium binding constant (K_{eq}) estimated with fluorescence titration is 12.32 × 10^5 M^{-1}, which is about 3-fold of single-stranded DNA (da21), double-stranded DNA (ds26) and G4-DNA (htg21). In addition, the fluorescence quantum yield (Φ_f) of the RNA-bound dye determined is 0.0562 relative to fluorescein while the free ligand is 0.0016, indicating that the RNA-dye complex formed *in situ* may suppress the intrinsic non-radiative relaxation in terms of molecular vibration or rotation.[53] The ligand also exhibits excellent photostability, cell tolerance, and counterstain compatibility with 4',6-diamidino-2-phenylindole (DAPI) for specific RNA–DNA colocalization in live cell imaging assays with PC3, HUVEC, NIH-3T3 and L929 cells.[52a]

PI2 is a cell permeable and small-sized fluorescent ligand (λ_{ex} = 409 nm; λ_{em} = 511 nm) highly selective to RNA.[52b] The ligand is modified based on a fluorescent RNA dye E36[54]. An aminoethylpiperidine substituent group is introduced at the 4-position of 1-methylquinolinium scaffold of the ligand. Interestingly, the introduction of this polar and flexible aminoethylpiperidine substituent is able to enhance markedly both RNA-specificity and binding affinity (K_{eq} = 4.48 × 10^5 M^{-1}) compared to E36 (K_{eq} = 0.89 × 10^5 M^{-1}). In addition, the live cell imaging study with **PI2** in PC3 cancer cells exhibits strong enhanced fluorescence signal that may probably indicate the interaction of the ligand with RNA substrates in the regions of nucleolus and cytoplasm. The fluorescence quantum yield (Φ_f) of the RNA-bound dye was found to be 0.577 relative to fluorescein while the free ligand was 0.0039. For E36, the Φ_f of RNA-bound dye determined under the same conditions was 0.114 and the free ligand was 0.0022. Moreover, the water solubility of **PI2** is 0.216 mg/ml, which is almost 2-fold of E36 (0.110 mg/ml). In addition, in live cell imaging study, **PI2** examined in PC3 cancer cells shows higher photostability than E36. Furthermore, the cytotoxicity of **PI2** evaluated with

3T3 cells using MTT assays shows that the cell viability is greater than 85% for 12 h with the ligand at 20 μM. These results suggest that **PI2** at the concentration lower than 20 μM may has very low or no cytotoxicity to the cells examined.[52b]

Probe 1 reported by Yi and co-workers,[49b] is synthesized based on a 1,8-naphthalimide scaffold and is nuclear membrane permeable (staining the nucleolus in the cells). The water solubility of the ligand could be up to 10 mM in phosphate buffer. The ligand was evaluated in the detection of intracellular rRNA in HeLa cells and it exhibited a 32-fold fluorescent enhancement upon binding to rRNA (λ_{ex} = 488 nm, λ_{em} = 532 nm) in phosphate buffer. It also showed good selectivity toward rRNA against DNA and other nucleic acids including tRNA. For the molecular design, it was found that a flexible ethylamine substituted at the N-position and a small dimethylamine group substituted at the 4-position of the naphthalimide scaffold could give better rRNA interaction and penetration ability toward the cellular membrane and nuclear pores of HeLa cells. These two substituent groups result in the ligand showing an appropriate lipophilic balance and this property renders the molecule showing excellent cellular membrane permeability. In addition, the fluorescence response of the ligand to rRNA interaction is proposed to be a typical twisted ICT (TICT) mechanism, in which the dimethylamine group of the ligand is rigidified upon interacting with rRNA substrates. The rRNA-ligand interaction suppresses the TICT of the ligand and results in the generation of fluorescence signal. Moreover, the cytotoxicity of the ligand was evaluated by MTT assays with HeLa cells. It was found that the ligand (up to 30 μM) exhibited more than 90% cell viability after 6 h of incubation with the cells. The photostability of the ligand was also found better than that of SYTO RNAselect™ under irradiation at 620 nm for 150 min.[49b]

QUID-2, developed by Tan and Chen and co-workers, is an RNA-selective small molecule ligand reported most recently.[49c] The molecular structure of **QUID-2** adopts a cationic and planar 1-methylquinolinium scaffold that is also utilized in **Styryl-TO** and **PI2**. Interestingly, these three RNA-selective ligands apply a flexible ethylene bridge at the 2-position of 1-methylquinolinium scaffold to conjugate with a substituent group, such as styrene and indole, to build classical styryl probes with novel molecular structures. This approach for ligand design remarkably improves the selectivity and sensitivity toward RNA targets *in vitro* and *in cellulo*. The limit of detection of **QUID-2** for the RNA was found down to 1.8 ng/mL in buffer. In addition, **QUID-2** was demonstrated for RNA imaging in live HeLa cells (λ_{ex} = 488 nm, λ_{em} = 500–600 nm). The dynamics of RNA and RNA granules formation in live cells could also be monitored with the ligand. Moreover, the ligand at a concentration 40 μM or lower exerted negligible toxicity against HeLa cells. **QUID-2** also shows better photostability than SYTO RNASelect™ in live cell imaging under continuous excitation at 488 nm for 5 min.[49c]

For the RNA-selective probes, **Styryl-TO**, **PI2**, **Probe 1** and **QUID-2**, demonstrated in live cell imaging, it could be generally concluded that confocal fluorescent images may confirm the cell membrane permeability of the dye and their intracellular localization in the nucleolar region and/or the perinuclear portion of cytoplasm. Moreover, RNase and DNase digestion assays are the commonly used protocol to validate the intracellular binding target of the ligand is RNA but not DNA. Some selected examples are shown in **Figure 4** for illustration.

As a biosensor for imaging and real-time monitoring of the RNA target of interest in live cells, apart from the selectivity and sensitivity of the probe, the water solubility, cytotoxicity, fluorescence quantum yield and intracellular photostability are important parameters determining the performance of the probe in live cell imaging. It seems that most RNA-selective ligands developed are excited in the visible light region (400–600 nm).[20a, 20b, 22a, 22d] However, for *in vivo* studies, the tissue penetration ability of the light source required to excite the ligand may be an important factor of consideration. Over the last two years, despite some other RNA-binding fluorescent ligands or sensors have been demonstrated capable of imaging RNA substrates in live human cells such as MCF7 and A549 cell,[55] the fluorescence microscopic images obtained from RNase and DNase digestion assays suggest that these ligands may not selectively target RNA under intracellular conditions.

Biosensing and imaging of RNA G-quadruplex (G4) structures in live cells are also an emerging hot research area because the *in vivo* existing and biofunction of these special noncanonical secondary nucleic acid structures are still unclear.[30, 56] The RNA G4-selective fluorescent ligands with the use of advanced fluorescence microscopy techniques may provide a powerful tool for in-depth investigations. Currently, there are just a few *X*-ray crystal structures of RNA G4 structures reported and thus their structural information is very limited at present.[20a, 57] It is therefore an challenge task for molecular chemists to design highly selective fluorescent ligands targeting RNA G4-structures. Nonetheless, about fourteen fluorescent ligands shown in **Figure 5** have been reported currently and most of them have been reviewed over the past two years.[20a, 20b, 46b]

TCB-1[58] and **BYBX**[49d] are the highly RNA G4-selective and small-sized fluorescent ligands disclosed most recently. Interestingly, both ligands adopt benzothiazole as a scaffold in the molecular design and also are bridged with a planar cationic moiety, such as

quinolinium and benzoindolium, via a relatively rigid methylene bridge. Among the RNA G4-selective fluorescent ligands reported (**Figure 5**), the scaffolds of benzothiazole, indole, *N*,*N*-dimethylaniline, quinolinium and benzoindolium are most frequently utilized for the design of RNA G4-selective ligands. **TCB-1**, an analogue of thiazole orange, reported by Tang and co-workers in 2022,[58] shows good selectivity binding to RNA G4-structures. The G4-**TCB-1** interaction in buffer solution enhances 150- to 438-fold of fluorescence (λ_{ex} = 480 nm, λ_{em} = 526 nm), while other nucleic acid substrates could only enhance 30-fold or less fluorescence signal under the same conditions. **TCB-1** was able to avoid lysosome capture and thus it could evenly disperse in cytoplasm and selectively lighted up the cytoplasmic RNA G4-structures. However, the ligand is not cytoplasm-specific as it also stains nucleoli. The intracellular binding target of **TCB-1** was validated with an immunofluorescence assay using G4-specific antibody BG4, in which the fluorescence foci of the ligand were well co-localized with BG4. Moreover, the ligand was demonstrated to detect the formation of cytoplasmic RNA G4 structures, which were formed from the overexpressed GGGCCT repeating unit in the NOP56 gene in live SCA36 model cells (**Figure 6A**). **TCB-1** is thus a potential RNA G4-biosensor for the identification of autosomal dominant spinocerebellar ataxia (SCA), which is a group of heterogeneous neurodegenerative diseases.

BYBX reported in early 2023 is a cytoplasm-specific fluorescent ligand.[49d] It is highly selective targeting RNA G4-structures including *VEGF*, *NRAS*, *BCL2* and *TERRA*, which are human cancer hallmarks. The fluorescence of **BYBX** (λ_{ex} = 447 nm, λ_{em} = 538 nm) were remarkably enhanced when it interacted with the RNA G4-structures (54-70 folds enhancement). Nonetheless, rRNA and RNA hairpin only showed mild fluorescence enhancement (14-20 folds) and non-G4 nucleic acid substrates exhibited almost no fluorescence. The binding affinity of **BYBX** toward various RNA G4 structures estimated with isothermal titration calorimetry (ITC) assays was in micromolar level (K_D = 2.5-3.3 μM), while non-G4 substrates exhibited either low or no binding affinity with the ligand. The selectivity of the ligand targeting G4-structures *in cellulo* was also validated by intracellular competition studies with BRACO-19 and PDS, and the colocalization study with a G4-specific antibody (BG4) in HeLa cells. The ligand was demonstrated in the visualization and monitoring of dynamic resolving process of RNA G4-structures by the overexpressed RFP-tagged DHX36 helicase in live HeLa cells. In addition, the photostability of the ligand was demonstrated in live cell imaging. The green foci of **BYBX** in live HeLa cells were able to keep steady over 60 min under irradiation at 488 nm. The results suggest that ligand could be robust against photobleaching. Moreover, the cytotoxicity of the ligand was evaluated against a number of human cancer cell lines including HeLa, HCT116, MDA-MB-231 and HFF1 by MTT assays. It was found that **BYBX** generally exhibited low toxicity against all these human cells tested (IC_{50} > 53 μM). This cytoplasm-specific and non-toxic small fluorescent ligand may provide a target-selective fluorescent tool for the real-time investigation of cellular biofunctions of RNA G4-structures associated with cancer for drug discovery.

4. RNA Structure-targeting Small Molecule Ligands for Drug Discovery

Conventional therapeutics using small molecule drugs mostly target proteins and have currently encountered the challenge of limited druggable targets. However, a large portion of disease-related proteins, about 85% of the human proteome, are considered undruggable because these proteins do not have distinctive clefts or pockets for interaction with small-molecules.[59] Alternatively, many researches have attempted to develop selective RNA-targeting small molecules as the drug and now the field is rapidly evolving. Since the identification of bioactive RNA-targeting small molecules such as the FDA-approved risdiplam, the biopharmaceutical industry has gained confidence in the direction of RNA-drug development.[34a] In addition, a number of recent review papers published over the last two years[21, 60] have comprehensively discussed the discovery of RNA structure-targeted small molecules utilizing various advanced experimental and computational technologies.

There are a number of potential targets from the functional site of different RNAs available for interacting with small molecules, for example, Drosha and Dicer processing sites within miRNA precursors, IRESs in viral RNAs and some human mRNAs, bacterial riboswitches, splicing enhancers and silencers in pre-mRNAs, and regulatory structures in 5'- and 3'-untranslated regions (UTRs).[21] In addition, the human genome produces a significant portion of non-coding RNA that may have roles in causing human diseases and thus the noncoding RNA could be also potential drug target of small-molecules.[61] Currently, different RNAs with themselves serving as either a drug (termed RNA therapy)[62] or a drug target, showing great potential as new therapeutics, have also been reviewed intensively.[60c, 60e, 60f, 60h]

Some small-molecules are able to occupy the functional site of RNA targets with strong affinity and capable of exerting biological effects in the interruption of downstream biology. Small molecules interacting with a variety of RNA targets, including those from human, bacteria and virus, that implicate in different human diseases including cancer, neuromuscular and

neurodegenerative diseases, and infectious disease, were well-summarized and discussed in two recent review articles published in 2022 (reviewed by Batey, Disney and co-workers)[21] and 2023 (reviewed by Morishita)[60a], respectively. At present, there are more than two hundred RNA-targeting small molecules reported.[63] These RNA-binding ligands could be broadly grouped into two categories regarding to their molecular mass: (i) molecular mass about 500 Da, the drug-like small molecule with the property satisfying Lipinski's rules based on the pharmacokinetic drug property; (ii) molecular mass about 2000 Da, the larger multivalent ligands. A platform for the sequence-based design of small molecules targeting different structured RNA molecules was established by Disney and co-workers in 2016.[64] The lately developed RNA structures targeting small molecules are discussed concentratedly in the following.

A number of drug-like small molecules bind specifically to the precursor of the oncogenic and pro-inflammatory microRNA-21 were reported by Varani and co-workers in 2023.[65] These anti-proliferative activity ligands, an analogue **52** shown in **Figure 7**, exhibit strong binding activity with mid-nanomolar affinity toward microRNA-21 and induce distinctive structural changes in the RNA that is correlated with a specific inhibition of miRNA processing. In addition, the cellular proliferation and microRNA-21 levels in gastric adenocarcinoma (AGS) and pancreatic (ASPC1) cancer cells were reduced with the ligand while kinases or classical receptors were not inhibited. The study demonstrates the feasibility of direct targeting mature miRNA sequences with small molecules and may provide new insights into the therapeutic development in multiple diseases where the oncogenic microRNA-21 is abnormally expressed.

Dovitinib, a receptor tyrosine kinase (RTK) inhibitor, is also rationally reprogrammed for pre-miR-21 that causes cancer, kidney disease and cardiovascular disease by using the drug as an RNA recognition element in a chimeric compound that recruits RNase L to induce catalytic degradation of RNA. This reprogramming of protein-targeted small-molecule drugs to RNA by ribonuclease recruitment was developed by Disney and co-workers in 2021.[66] The study demonstrated that the modification of Dovitinib into a RIBOTAC degrader (**Dovitinib-RIBOTAC**) was able to shift its selectivity from canonical RTK targets to the reprogrammed RNA target by 2500-fold with decreased toxicity that was verified in mouse models of disease, metastatic breast cancer and Alport Syndrome. The study may establish a foundation for reprogramming other known drugs for disease-associated RNA targets.

The highly conserved HIV-1 transactivation response (TAR) element is also considered as an attractive and promising RNA target for small molecules in the development of new anti-HIV therapies because this small RNA is capable of binding to Tat protein and is essential for HIV replication. The effective inhibition of Tat/TAR interaction with a potent ligand may suppress viral replication. A series of verapamil derivatives, reported by Duca and co-workers,[67] was demonstrated as the TAR-selective ligand. One of these analogues (**2h**) bearing an indole substituent group was found efficiently inhibiting Tat/TAR interaction *in vitro* (IC_{50} = 18.8 μM). The molecular mechanism of interaction was investigated with NMR and molecular docking. It was suggested that the ligand most likely interacted with the bulge site of TAR. This work illustrated a strategy to develop potential antiviral ligands targeting HIV-1 TAR RNA via structural modification of existing drugs such as verapamil.

Another small molecule ligand targeting TAR RNA was reported by Dash and co-workers in 2020.[68] The study demonstrates a rapid discovery of HIV-1 TAR-Tat binding ligands by the TAR RNA guided *in situ* cycloaddition reaction of azide and alkyne fragments. A thiazole-based peptidomimetic (**3ba**) bearing two thiazole scaffolds was discovered as a HIV-1 TAR-Tat inhibitor. The biophysical experiments including isothermal calorimetric (ITC), fluorescence and displacement studies reveal that the ligand may interact with the bulge region of TAR RNA (K_d = 0.49 μM) *in vitro* and thus inhibit Tat-TAR RNA interactions. Molecular docking study for **3ba** binding to the RNA target (PDB: 1ANR) revealed that anti-**3ba** could interact with the bulge region of the target. In addition, the flexible -CH_2- linker connected to the first thiazole ring of the ligand may make the molecule favourable to interact with the bulge region of TAR RNA.

RNA-repeat expansions are known to cause a number of microsatellite disorders and r(CCUG) repeats in Myotonic Dystrophy Type 2 (DM2) are also identified as a promising RNA drug target of small molecules. A recent study reported by Disney and co-workers in 2021 discovered that some drug-like small molecules capable of binding to the structure adopted by the expanded repeating nucleotides of r(CCUG) improved DM2-associated defects.[69] The compound **63**, optimized from screening hits that was obtained from an RNA-focused compound library, showed specific interaction with the RNA structure formed by the expanded r(CCUG) repeats with strong binding affinity (K_d = 192 nM). This molecular interaction facilitates the endogenous degradation of the aberrantly retained intron of the expanded r(CCUG) repeats through endogenous RNA decay mechanisms and rescues alternative splicing defects in DM2 patient-derived fibroblasts. It is noteworthy that the modification of the lead compound with a 2-methylbenzothiazole substituent group is important compared with its analogues, as indicated by a 20-fold improvement *in*

vitro activity and 5-fold improvement in cellular activity in DM2 patient-derived fibroblasts. This study demonstrates a targeted degradation of RNA through RNA quality control mechanisms and may be also applicable with monomeric RNA structures that could be recognized by drug-like small molecules.

The expanded repeat [r(CUG)^{exp}] is found causes myotonic dystrophy type 1 (DM1) and is recognized as an important RNA target of small molecules. Disney and co-workers has demonstrated recently that this RNA repeat is able to catalyze the *in situ* synthesis of inhibitor in DM1 patient-derived cells using an fluorogenic RNA-templated tetrazine ligation.[70] The on-site synthesized compound was found able to improve DM1-associated defects at picomolar concentrations. Moreover, this r(CUG)^{exp}-templated fluorogenic tetrazine ligation is able to synthesize an imaging probe on-site for the visualization of the RNA repeat in its native context in live cells and muscle tissue.

In addition, a benzimidazole derivative (**CB096**), targeting an RNA repeat expansion causing genetically defined c9ALS/FTD that triggered by RNA hexanucleotide (GGGGCC) repeat expansions (r(G_4C_2)) within the *C9orf72* gene, was reported by Disney and co-workers.[71] The **CB096**-RNA interaction studied with 1D imino proton NMR spectroscopy showed that the molecule interacted with the r$(G_4C_2)_8$ hairpin and altered the dynamics of the 5'CGG/3'GGC site within the RNA repeat. Molecular dynamics simulations study for the molecular recognition of the expanded repeating nucleotides of r(G_4C_2) by **CB096** was also performed and revealed that the NO_2 group substituted at the 5-position of benzimidazole and the 2-methoxyphenol moiety of molecule exhibiting important interaction with the 5'CGG/3'GGC motif within the expanded RNA repeat (r(G_4C_2)) hairpin. Furthermore, **CB096** is able to improve nucleocytoplasmic transport, inhibit the repeat associated non-ATG translation and reduce the stress granule formation in r$(G_4C_2)_{66}$-expressing HEK293T cells. The G4-structures of the (GGGGCC) repeat RNA, which is a hallmark of *C9orf72* disease, are also recognized as attractive drug targets of small molecule ligands. The recent discovery has demonstrated that ligands selectively bind to *C9orf72* repeat RNA G4-structures may reduce the levels of the damaging dipeptide repeat proteins in both the *C9orf72* patient-induced pluripotent stem cell-neurons.[72]

RNA G4-structures formed in the mRNA transcript of the human Kirsten RAS (*KRAS*) and Neuroblastoma RAS (*NRAS*) oncogenes are potential anticancer drug targets for G4-binding small molecules because the guanine (G)-rich 5'-UTR sequence of the mRNA harbouring several G4-motifs that form stable RNA G4-structures. Nonetheless, only few studies have been reported on this area currently. A small molecule ligand, 4,11-bis(2-aminoethylamino)anthra[*2,3-b*]furan-5,10-dione (**2a**),[73] reported by Xodo and co-worker, was found able to interact with RNA G4-structures in the *KRAS* transcript under low-abundance cellular conditions. Moreover, dual-luciferase assays demonstrated that the ligand repressed translation in a dose-dependent manner. The cellular effect of the ligand on Panc-1 cancer cells was also examined. The ligand was found cell membrane permeable and suppressed the protein p21KRAS to lower than 10% of the control. The effective down-regulation of p21KRAS expression by the ligand induces apoptosis and reduces cell growth and colony formation. The study demonstrates an attractive strategy to suppress the *KRAS* oncogene in pancreatic cancer cells with small molecules binding to RNA G4-structures formed in the 5'-UTR of mRNA.

A NRAS RNA G4-targeting ligand (**4a-10**),[74] developed by Ou and co-workers, was designed with a rigid and planar quindoline scaffold substituted with a *p*-(methylthio)styryl group that was able to enhance the binding specificity and affinity (K_D = 0.7 μM) of the ligand toward NRAS RNA G4-structures. The strong interaction of the ligand with the G4-structure caused the repression of translation of the *NRAS* gene, which was validated with Western blot assays for NRAS proteins produced in A375 cells treated with the ligand. In addition, the ligand causes cell cycle arrest at G0/G1 phase and exhibits high cytotoxicity against a number of human cancer cells (IC_{50} = 1.1 to 8.8 μM, determined by MTT assays).

Apart from the RNA-targets discovered from the human genome, some viral RNA structures, such as the RNA G4-structure, are the emerging drug target of small molecules for the development of antiviral drugs currently. A recent study reported by Zhang and Li and co-workers[75] reveals that some classical G4-ligands including TMPyP4 and PDS inhibit porcine epidemic diarrhea virus (PEDV) proliferation in Vero cells (inhibitory rate over 99 % at 25 μM) and also inhibit infection independent of viral strains and cell lines because these G4-ligands reduce both virus genome replication and N protein expression. From 2022 to present, there are a number of studies reported on utilizing G4-ligands to target RNA G4-structures formed in SARS-CoV-2, a coronavirus, for COVID-19 treatment. In general, two strategies are applied currently to search for potent ligands targeting viral RNA G4-structures in the virus: (i) screening from reported G4-ligands but this approach may be very limited because there are only a few thousands G4-binding ligands reported thus far. Most of them are G4-DNA specific or selective while only few ligands selectively targeting RNA G4-structures (**Figure 5** and **Figure 7**); (ii) targeting RNA G4-structures with repurposed drugs. Repurposing of approved drugs is currently one of the most attractive propositions in drug development because this approach is to search bioactive compounds

from the “de-risked” approved drugs. More importantly, the drug repurposing strategy may possibly lower the cost and shorten the development timelines for new drugs despite there are still some challenges ahead.[76]

The COVID-19 pandemic caused by SARS-CoV-2 has become a global threat over the past few years. Several G-rich sequences of SARS-CoV-2, capable of forming stable G4-structures *in vitro*, have been identified in the coding sequence regions of nucleocapsid protein, non-structural protein 10, and non-structural protein 3. Some well-known G4-struture stabilizers including PDP (pyridostatin derivative)[77], TMPyP4[78] and Berberine[79] were evaluated for the development of effective antiviral agents against COVID-19. Furthermore, some clinical approved drugs including Netarsudil[80], Topotecan and Berbamine[81] have also identified by *in silico* and *in vitro* assays as potent stabilizers targeting RNA G4-structures with repurposing potential against COVID-19. All these recent studies generally disclose that the potent G4-ligands screened may bind to and stabilize the RNA G4-structures formed in the virus and the strong interaction results in the inhibition of viral replication and translation both *in vitro* and *in vivo*. The antiviral activity is therefore achievable with potent G4-ligands.[82]

5. Summary and Outlook

In recent years, RNA G4-structures have become an emerging nucleic acid target for chemical biology and drug discovery studies because these noncanonical secondary structures may play critical roles in the regulation of RNA splicing, transport and translation, and RNA-mediated stress responses in cytoplasm. Compared to DNA G4-structures, small molecule ligands selectively targeting RNA G4-structures are extremely underexplored. Up to now, RNA and G4-RNA structures selective ligands developed may be just less than hundred while that of DNA has a few thousands found in literature. One of the possible reasons could be due to the challenge that RNA structures are diverse and we now have very limited RNA structural information. High-resolution NMR or *X*-ray crystal structures of RNA targets of interest for ligand design are mostly unavailable currently. High-throughput screening and computer-aided drug design are both supportive tools for searching bioactive small molecules as the lead compound for further structural advancement and in-depth structure-activity relationship investigations.

The recent achievements on the research field targeting RNA structures for drug development, including but not limited to those selectively discussed in this review, due to limited space, demonstrate that RNA structures, alternative to the dominated protein-based drug targets, could be the promising targets of small molecules for drug discovery against various human diseases, particularly anticancer, antibacterial and antivirus.

In addition, it is well-known that mRNA-targeted small molecules may regulate the synthesis of disease-related proteins. The non-covalent mRNA-ligand interaction does not involve gene modification and this approach may be a feasible alternative for the “undruggable” proteins, such as RAS proteins (anticancer drug targets) and bacterial FtsZ protein (an antibacterial drug target), with their enzymatic activity hard to be suppressed with small molecule ligands. These ligands could be also designed specifically localized in cytoplasm for interacting with RNA targets but non-permeable to nuclear membrane (not localized in nucleus). Thus, these tailored ligands may possibly minimize the competitive DNA interaction in live cells.

Moreover, to understand better the mechanism of RNA-ligand interaction and their cellular biological effects, the real-time visualization of RNA targets of interest in live cells and/or *in vivo* is critically important. The development on this research direction is still a relatively new area of study because only very limited numbers of RNA-target selective fluorescent ligands have been reported thus far. Despite advanced microscopy techniques, such as live cell confocal microscopy, are available, the real-time intracellular study requires sensitive, selective, photostable, low-toxic, cell membrane permeable and long wavelength absorption and emission probes.

Furthermore, it is a great challenge in molecular design without knowing the 3D structure of an RNA target. In addition to obtaining *X*-ray crystal structures for the RNA target of interest, molecular docking and simulation, high-resolution NMR spectroscopy and cryogenic transmission electron microscopy (cryo-TEM) are the indispensable tools to validate the interaction of a ligand with the RNA target.

Taken together, the current experimental biochemical and biophysical characterizations and findings have demonstrated that RNAs are druggable targets for small molecules. Nonetheless, one of the critical challenges is that our understanding on both structure and function of RNAs is still very limited. With the use of contemporary chemical probing techniques could enable the profiling of RNA–ligand binding interactions transcriptome-wide; however, both the mechanism and mode of interactions are difficult to be fully understood. We require more structural information of RNA targets because it allows tailor-made molecular design to interrupt the function of RNA targets with high specificity, affinity and potency. Thus, we need to enrich largely the

currently limited ligand library for drug screening and in-depth structural-activity relationship investigations. Furthermore, mRNAs are definitely important drug targets of small molecules for RNA therapeutics development as they encode proteins; however, a majority of human genome sequences is transcribed as functional ncRNAs. The amount of ncRNA is largely outnumbered mRNAs. Thus, it is also a vital target that cannot be overlooked for new ligand development. It is expected that, as more researchers contributing their efforts in this area, most challenges encountered currently will be resolved and more potential RNA-binding ligands can reach clinical trials. Hopefully, RNA-targeted small molecule drugs can be realized for therapeutic treatments against human diseases in the foreseeable future.

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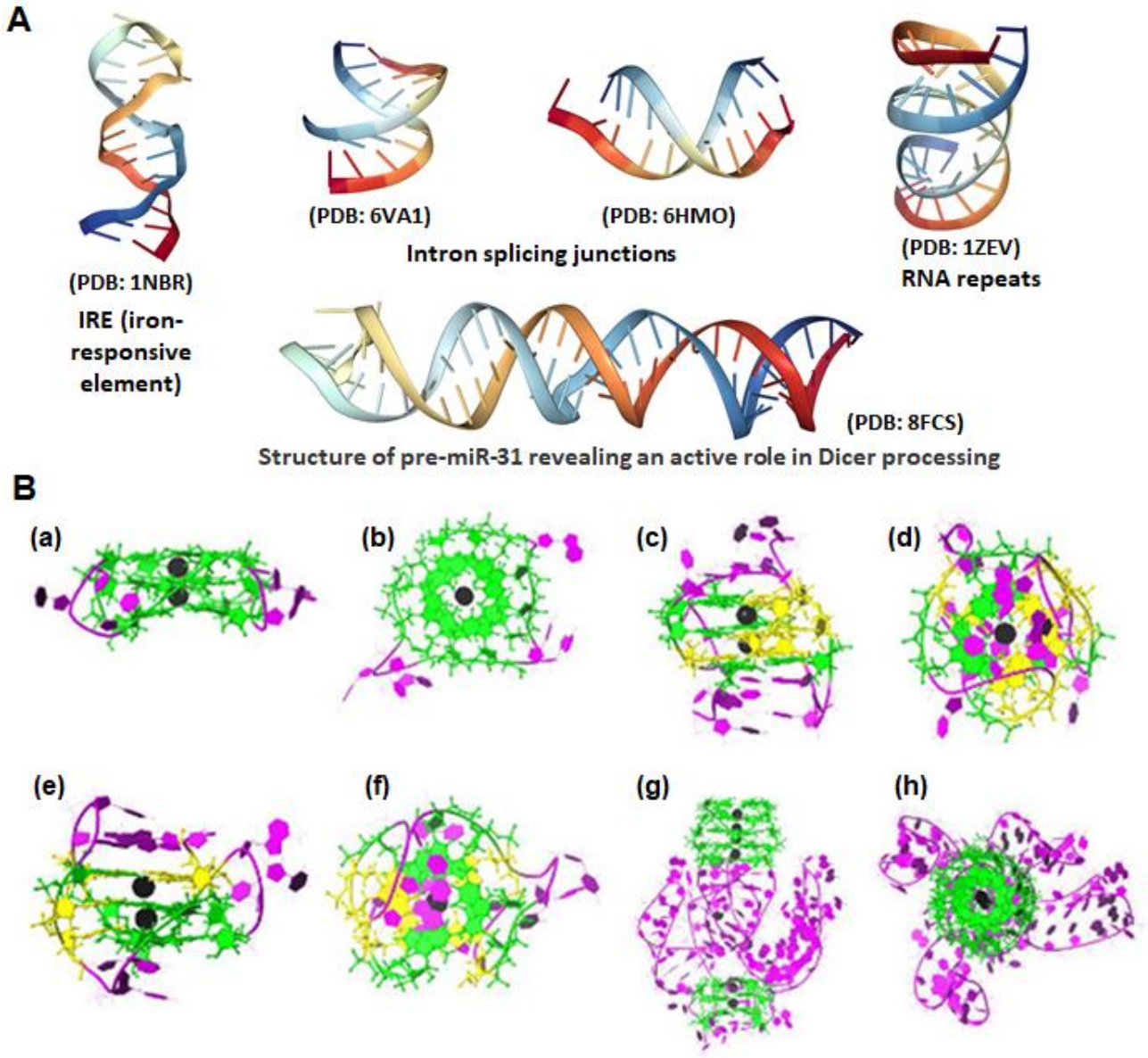

Figure 1. (A) Examples of human RNA structures regulating key biological functions: IRE (iron-responsive element; translational regulation; PDB: 1NBR). Splicing modulators (alternative pre-mRNA splicing including those interact with U1 small nuclear RNA (snRNA); intron splicing junctions (PDB: 6VA1, 6HMO)). RNA repeat expansions (aberrant gain-of-function, microsatellite disorders. PDB: 1ZEV) and Drosha and Dicer processing sites in microRNA (miRNA) precursors (structure of pre-miR-31 revealing an active role in Dicer processing, PDB: 8FCS). (B) Selected models of RNA G4-structures based on structural predictions. (a) and (b): A three-tier parallel RNA G4 modelled for 5′-GGGAGGGGCGGGUCUGGG, lateral and top view, respectively; (c) and (d): An anti-parallel RNA G4, modelled for 5′-UUAGGGUUAGGGUUAGGGUUAGGGUUA based on analogous DNA G4 (PDB: 2MBJ); (e) and (f): A proposed hybrid RNA G4, modelled for 5′-UAGGGUUAGGGUUAGGGUUAGGG based on analogous DNA G4 (PDB: 2JSM). (g) and (h): A tetramolecular RNA G4, modelled for 5′-GGGGGUGUAGCUCAGUGGUAGAGCGCGUGC based on the sequence of 5′-tiRNA^{Ala}.[29]

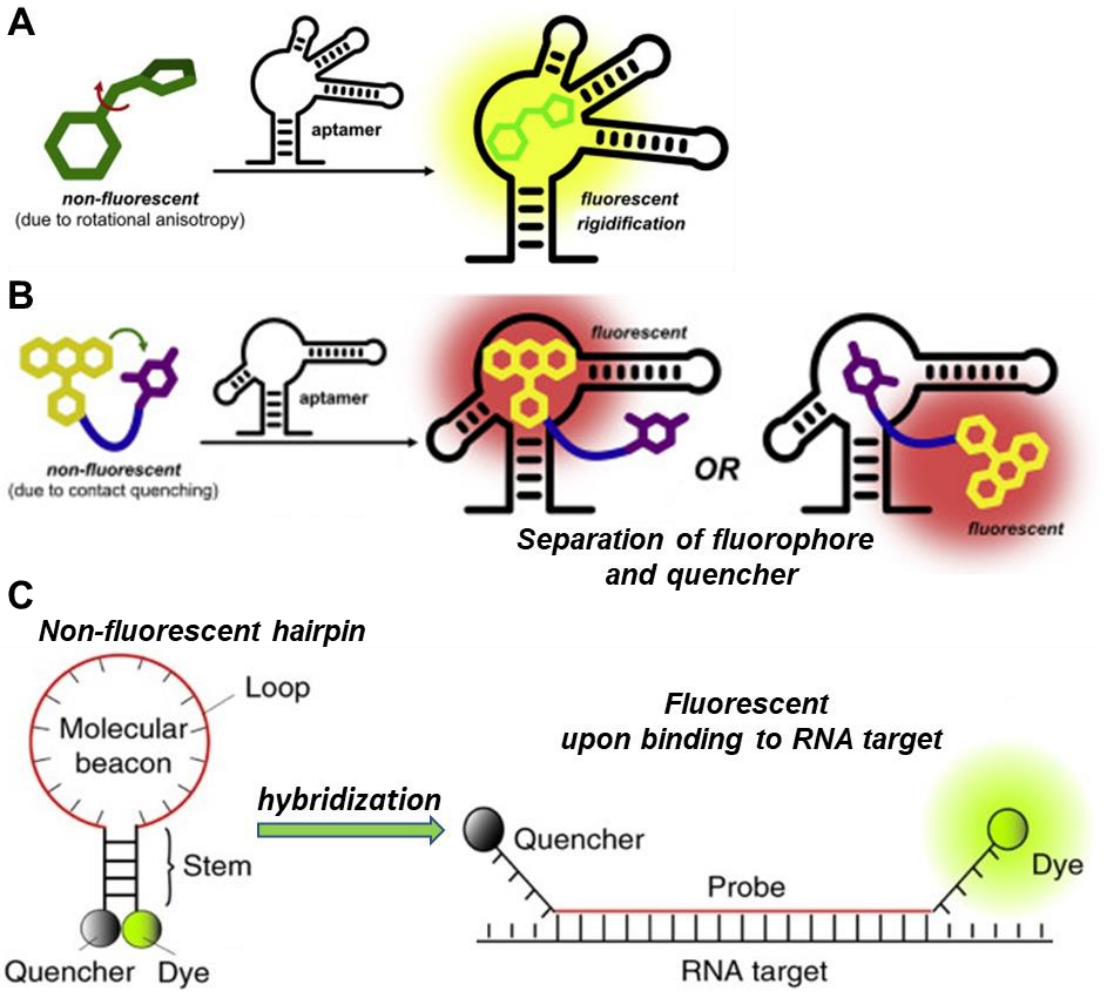

Figure 2. Models of fluorophore-aptamer pairs, fluorophore-quencher pairs and molecular beacons for RNA imaging. (A) The fluorophore-aptamer pairs generate fluorescence turn-on when the non-fluorescent molecule is rigidified by interacting with the aptamer.[22c] (B) The non-fluorescent tethered fluorophore-quencher pairs enable an aptamer binding event to separate fluorophore-quencher pairs and turn-on fluorescence.[22c] (C) An illustration of molecular beacons for RNA imaging. The non-fluorescent hairpin is a dual-labelled stem-loop oligonucleotide-based probe with a reporter fluorophore at one end and a quencher molecule at the other end. The designed oligonucleotide hairpin probe upon binding to the RNA target, the fluorophore and quencher are thus separated and turn-on fluorescence.

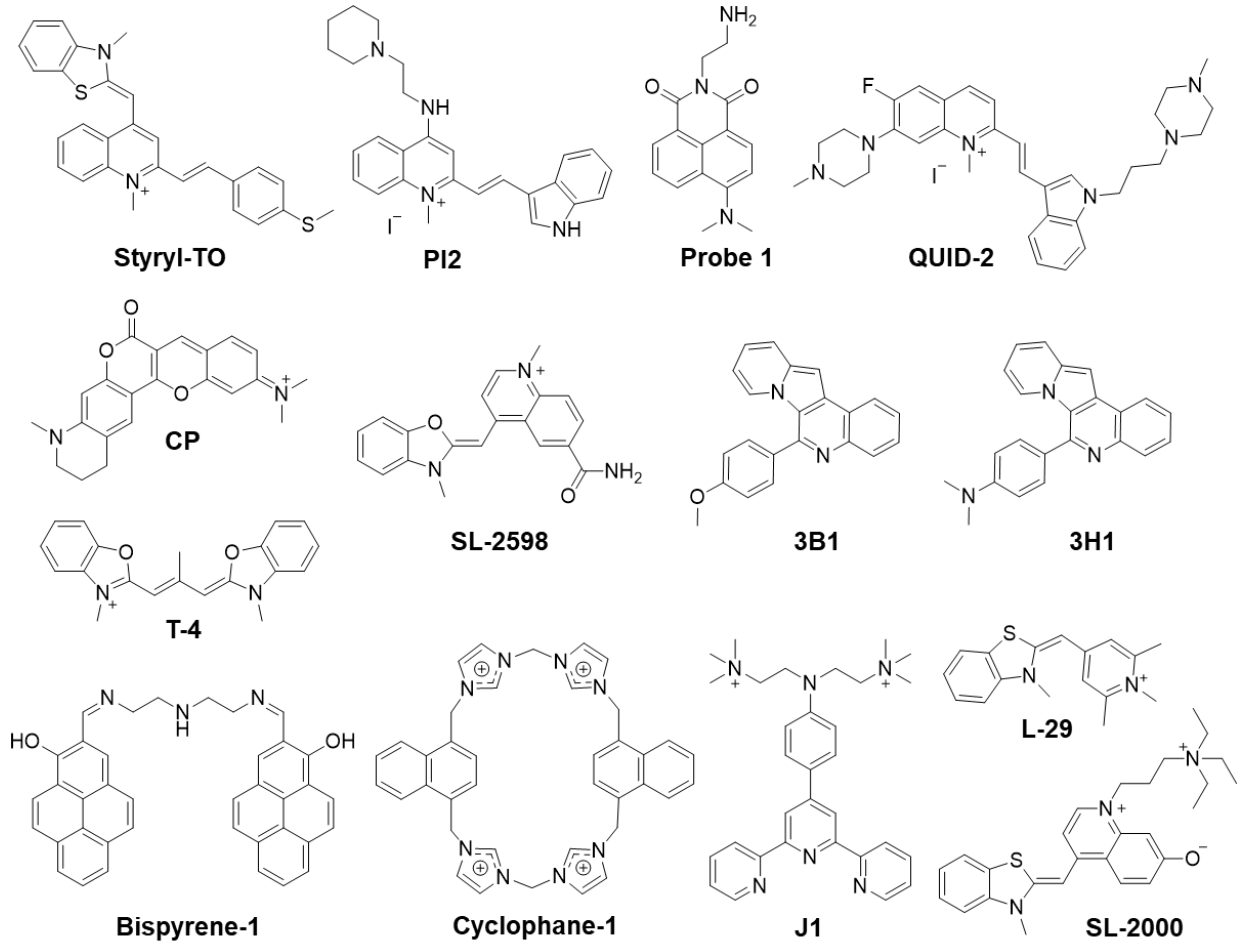

Figure 3. The recently reported small molecule-based fluorescent ligands selectively targeting RNA in live cells.

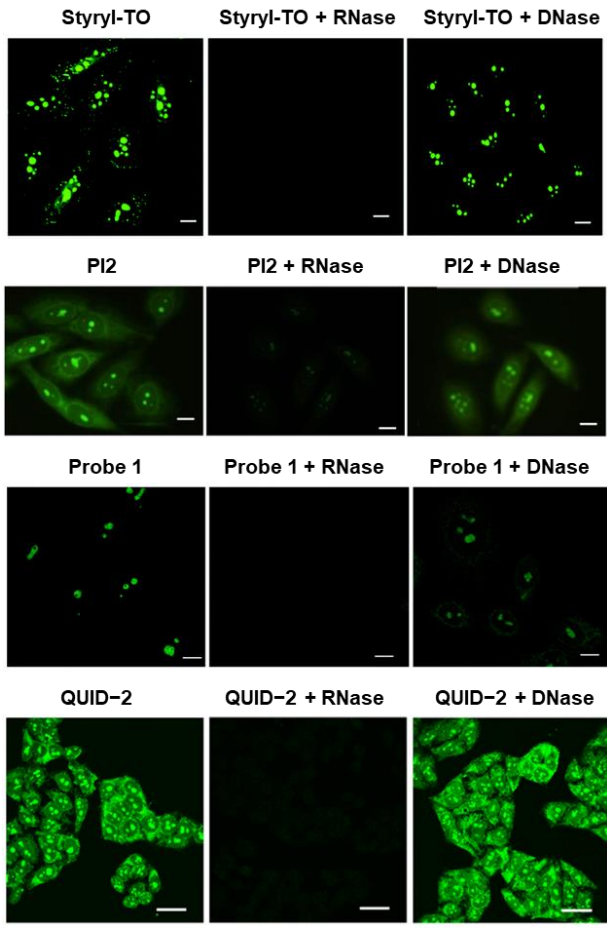

Figure 4. DNase and RNase digestion assays. The confocal images of **Styryl-TO**, **PI2**, **Probe 1** and **QUID-2** stained on different human cells including PC3 and HeLa cells.

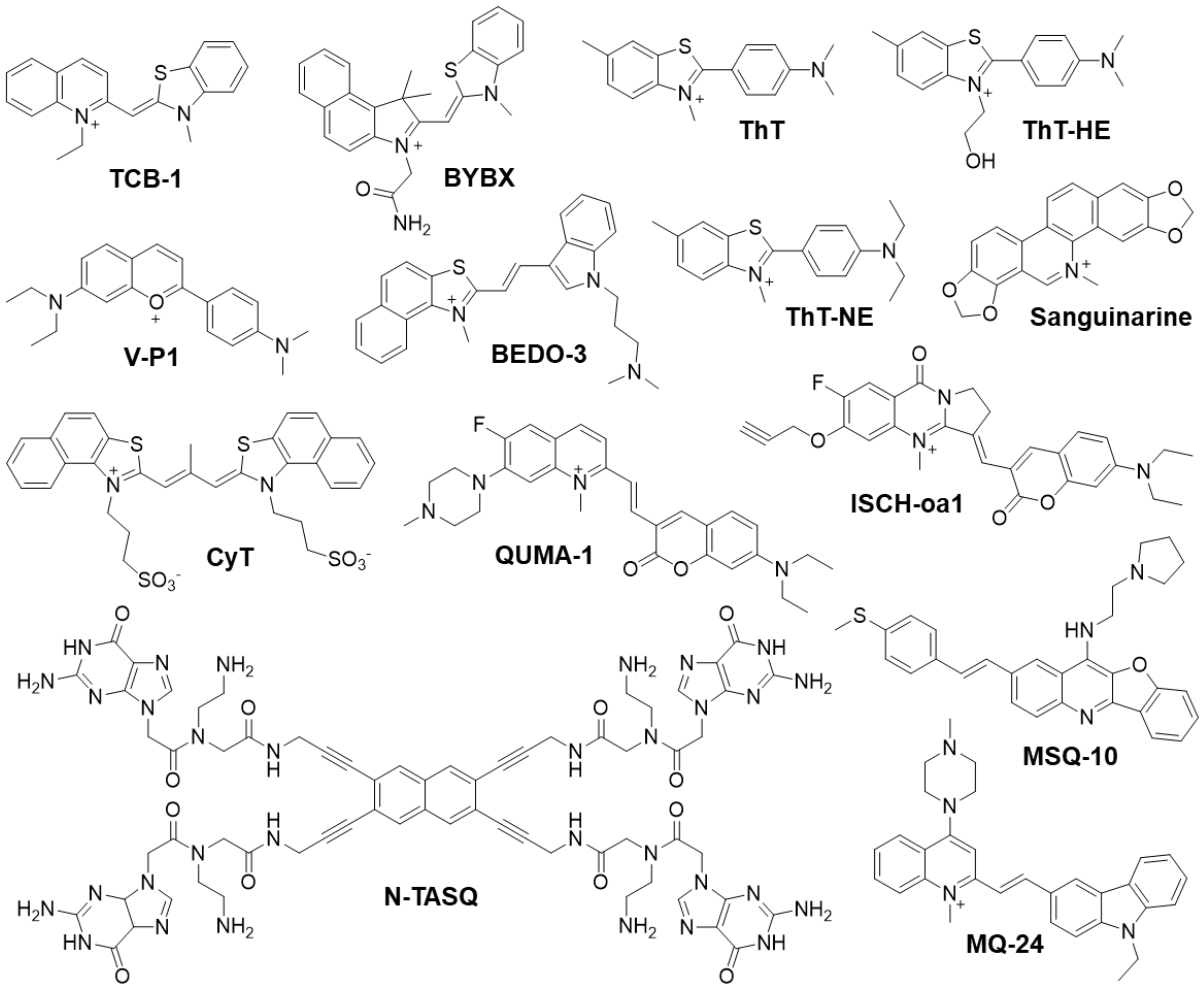

Figure 5. RNA G4-structure selective fluorescent ligands.

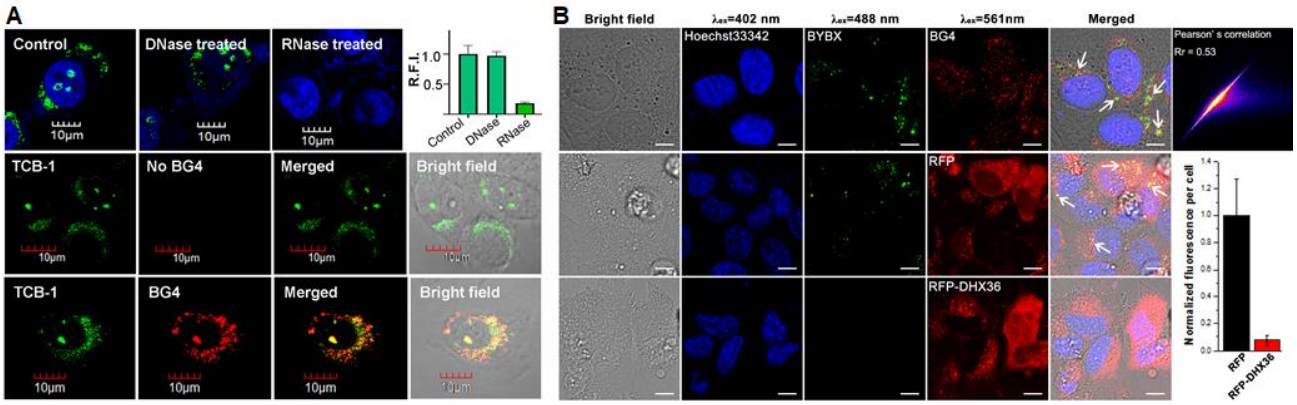

Figure 6. The most recently reported RNA G4-selective fluorescent ligands and their live cell imaging study targeting endogenous RNA G4-structures: (A) **TCB-1** in SCA36 model cells;[54] (B) **BYBX** in HeLa cells.[45d]

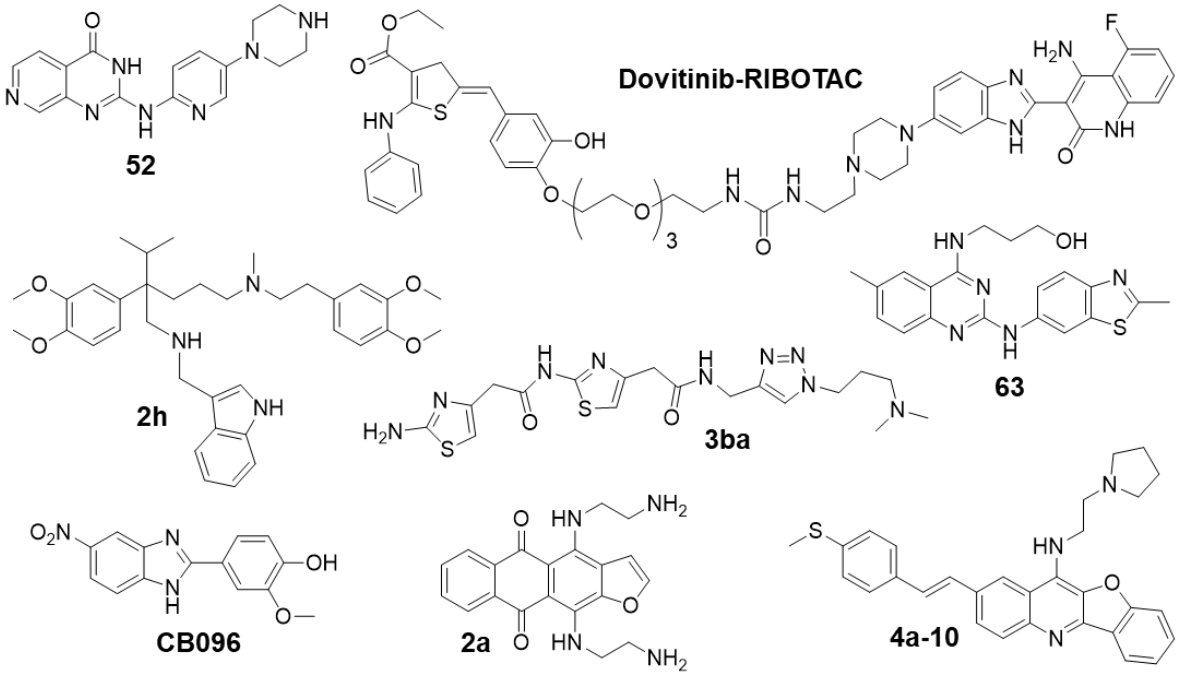

Figure 7. Recently reported bioactive small molecules selectively targeting RNA and RNA G4-structures.