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# Fragment-based drug discovery for RNA targets

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**Abstract:** Rapid development within the fields of both fragment-based drug discovery (FBDD) and medicinal targeting of RNA provides possibilities for combining technologies and methods in novel ways. This review provides an overview of fragment-based screening (FBS) against RNA targets, including a discussion of the most recently used screening and hit validation methods such as NMR, X-ray crystallography, and virtual screening methods. A discussion of fragment library design based on research from small molecule RNA binders provides an overview on both the currently limited guidelines within RNA-targeting fragment library design, and future possibilities. Finally, future perspectives are provided on screening and hit validation methods not yet used in combination with both fragment screening and RNA-targets.

Kasper Plenov Lundquist, born in Denmark 1996, studied chemistry at the Technical University of Denmark (DTU), where he obtained his MSc degree in 2020. Since then, Kasper has pursued a PhD degree within fragment-based drug discovery against nucleotide targets under the supervision of Professor Mads Hartvig Clausen (DTU), Associate Professor Charlotte H. Gotfredsen (DTU), and Professor Timor Baasov (Technion). His academic interests currently focus on medicinal chemistry and chemical biology.

During his doctoral study, Vipul Panchal focused on the pathogenesis of *Mycobacterium tuberculosis*, the causative agent of tuberculosis at the Institute of Genomics and Integrative Biology, India and received a PhD degree in 2018. In 2019, he joined the Brenk lab at the University of Bergen, Norway to apply structure-based drug discovery to explore novel targets for new generation of antibacterials. With Prof. Ruth Brenk currently he is exploring the therapeutic potential of riboswitch element(s) as an antibacterial target by developing riboswitch-specific high throughput and virtual screening techniques together with conventional structural biology approaches.

Charlotte Held Gotfredsen, born in 1967, studied Chemistry and cell Biology at SDU Odense where she obtained her PhD in 1998 with Assoc. Prof. Jens Peter Jakobsen and Professor Jesper Wengel. Charlotte was during her PhD a year (1995–1996) at UCLA with Professor Julie Feigon. From 1998–2004 Charlotte was a postdoctoral fellow at the Carlsberg Laboratory before joining DTU as an associate professor. In 2015, she became Head of the NMR Center • DTU. Her research focuses on NMR spectroscopy at the interface between chemistry and biology, chemical biology, biological chemistry, and NMR based screening.

Ruth Brenk is a pharmacist by training. In 2003, she obtained her PhD from the University of Marburg (Germany) under the guidance of Prof. Gerhard Klebe. Afterwards, she joined the group of Prof. Brian Shoichet at the University of California, San Francisco as a postdoc. In 2005, she became a Lecturer at the University of Dundee (UK). In 2012, she became Junior professor at the Institute of Pharmacy at the Johannes-Gutenberg University Mainz (Germany). In 2015, she was appointed professor at the University of Bergen (Norway) where she is leading a research group in structure-based drug design and molecular recognition.

Mads Hartvig Clausen, born in 1974, studied chemistry at the Technical University of Denmark (DTU), where he obtained his PhD in 2002 with Professor Robert Madsen. Mads was a postdoctoral fellow at Harvard University from 2002–2004 with Professor Andrew Myers before returning to DTU as an assistant professor. In 2014, he became full professor of chemical biology at DTU. His research focuses on chemical biology, medicinal chemistry, and library synthesis.

## 1. Introduction

More attention is being paid to RNA as a pharmaceutical target, and drug development methods usually focused on proteins are now being directed towards RNA as well, which includes fragment-based drug discovery (FBDD) techniques. While excellent reviews that cover important aspects of the use of FBDD against RNA targets have been published,[1–6] there is a need for a review of the most essential and recent FBDD screening methods against RNA and what future possibilities and new methods for these could be. Combined with an updated view on the current challenges and solutions in the field we hope this review will enable further development of FBDD for RNA.

### 1.1. RNA as a pharmaceutical target

RNA as a pharmaceutical target has historically been overshadowed by proteins, and most of the small-molecule drugs developed against RNA targets have been antibiotics targeting the prokaryotic ribosome.[6] Limitations of protein druggability have become more apparent as at least 67% of the proteins encoded in the human genome are considered undruggable due to them being intrinsically disordered proteins (IDPs).[4] Additionally, only about 1.5% of the human genome codes for proteins while more than 50% are being transcribed into RNA, suggesting that non-coding RNA (ncRNA) may provide a promising druggable alternative.[4,6] Examples of these are riboswitches which regulate the expression of downstream genes through binding of small molecules,[7,8] as well as tRNA and rRNA involved in the translation of proteins.[9] As more attention is paid to RNA as a target, technologies for screening and structural determination are also developing, providing new opportunities for targeting diseases through RNA instead of proteins.[5]

### 1.2. Fragment-based drug discovery

Parallel to the increase in popularity for RNA as a pharmaceutical target, FBDD has also seen an increase in use supported by technological development, with NMR spectroscopy, surface plasmon resonance (SPR), and X-ray crystallography being among the most popular screening methods.[10] The primary benefit from FBDD is the ability to cover the same or even an expanded chemical space with fewer molecules, compared to high-throughput screening (HTS), allowing for the use of fragment libraries consisting of only a few hundreds to a few thousands.[11,12] Additionally, while RNA-binding molecules often are rather large and contain multiple charged groups which may cause their interactions to be more promiscuous, FBDD can provide ligands with higher ligand efficiency and result in more specific binders.[1] These advantages of FBDD come at the expense of low affinities of the hit compounds necessitating subsequent optimization.

FBDD is well established for protein targets with an increasing number of compounds developed entering into various stages of clinical trials.[13] In contrast, implementing this methodology for RNA targets has just begun, which can be attributed to the late realization of RNA druggability. Multiple recent reviews have been published on the topic of RNA as a target for small molecules, with a review by Connelly *et al*. from 2016 covering most of the small molecule – RNA screening approaches including a section on FBDD,[5] while a more recent review from 2019 by Sztuba-Solinska *et al*. provides broader coverage of the current RNA targets and the most popular screening methods against them, also including a section on FBDD.[2] A book chapter from 2017 by Wehler and Brenk provides an overview of structure-based discovery of small molecules binding to RNA with a thorough section on FBDD against RNA,[3] and a 2020 review by Haniff *et al*. also offers insight into small molecule screening methods to study RNA binding, including a review of the increasingly popular virtual screening methods.[4]

Herein, we discuss various techniques that are increasingly employed to screen fragment libraries against RNA targets (Table 1). As validation and optimization of hit fragments is a critical part of FBDD, we have further extended the discussion on the importance of various techniques in identifying and guiding the chemical elaboration of hits to drug-like lead molecules. Further, recognizing the importance of the library composition to the success of a drug discovery program, we have reviewed the possible bias of RNA targets for fragments and have included recommendations and future perspectives for RNA focused fragment libraries. Finally, we end the review by introducing promising techniques awaiting evaluation for their suitability for RNA targeting fragment-based screening (FBS).

**Table 1.** An overview of the methods covered in the review, a short description of their use in the context of fragment screening, and a key reference describing the application of the method.

| Method | Use | Key references |
|---|---|---|
| RNA-observed $^{1}$H NMR | Identifies fragment hits either by changes in chemical shift of the $^{1}$H resonances in the RNA molecule or by a decrease in resonance intensity. Allows identification of hit-target interactions if the RNA structure is known. | **Lee *et al*:** *A novel small-molecule binds to the influenza A virus RNA promoter and inhibits viral replication.*[14] |
| Ligand-observed $^{19}$F NMR | Identifies fragment hits either by changes in chemical shift of $^{19}$F resonances in the fragment molecule or by a decrease in the resonance intensity when interacting with RNA. Allows for use of larger fragment cocktails but requires a library of fluorinated fragments. | **Binas *et al*:** *$^{19}$F NMR-Based Fragment Screening for 14 Different Biologically Active RNAs and 10 DNA and Protein Counter- Screens.*[15] |
| Ligand-observed $^{1}$H NMR | Identifies fragment hits either by changes in chemical shift of $^{1}$H resonances in the fragment molecule or by a decrease in the resonance intensity when interacting with RNA. Fragment cocktail sizes are limited compared to ligand-observed $^{19}$F NMR, but a bespoke fragment library is not required. | **Tam *et al*:** *Discovery of small-molecule inhibitors targeting the ribosomal peptidyl transferase center (PTC) of M. tuberculosis.*[16] |

| | | |
|---|---|---|
| Fluorescence-based competition assay | Identifies fragment hits by changes in fluorescence caused by release of a fluorescently labeled competitive binder. Provides information on hit-RNA interaction but requires a known binder. | **Zeiger *et al*:** *Fragment based search for small molecule inhibitors of HIV-1 Tat-TAR*.[17] |
| Radiolabeled competition assay | Identifies fragment hits by changes in radioactivity caused by release of a radiolabeled competitive binder. Provides information on hit-RNA interaction but requires a known binder and handling of radioactive material. | **Cressina *et al*:** *Fragment screening against the thiamine pyrophosphate riboswitch thiM*.[18] |
| Chem-CLIP-Frag-Map | Identifies fragment hits through covalent binding to $^{32}$P-labeled RNA targets and allows subsequent hit validation using cross-linking to stop reverse transcriptase. | **Suresh *et al*:** *A general fragment-based approach to identify and optimize bioactive ligands targeting RNA*.[19] |
| Virtual screening | Identifies fragment hits through docking against available RNA crystal and/or NMR-based structures. | **Daldrop *et al*:** *Novel ligands for a purine riboswitch discovered by RNA-ligand docking*.[20] |
| X-ray crystallography | Validates fragment hits by providing information on hit-RNA interactions through crystallographic structures. | **Warner *et al*:** *Validating Fragment-Based Drug Discovery for Biological RNAs: Lead Fragments Bind and Remodel the TPP Riboswitch Specifically*.[21] |
| SHAPE | Validates fragment hits by providing information on hit-RNA interactions through changes to target stability by chemical modification and cleavage. | **Warner *et al*:** *Validating Fragment-Based Drug Discovery for Biological RNAs: Lead Fragments Bind and Remodel the TPP Riboswitch Specifically*.[21] |
| Hit validation by NMR spectroscopy | Validates fragment hits by providing information on hit-RNA interactions through structure elucidation facilitated by various multidimensional NMR experiments. | **Lee *et al*:** *A novel small-molecule binds to the influenza A virus RNA promoter and inhibits viral replication*.[14] |
| Computational methods | Computational methods including docking, machine learning algorithms, and free energy calculations rely on structural data from RNA targets being available. They can provide information on target-ligand interactions which is critical for further development of fragment hits against RNA targets. | **Tam *et al*:** *Discovery of small-molecule inhibitors targeting the ribosomal peptidyl transferase center (PTC) of M. tuberculosis*.[16]<br>**Tanida *et al*:** *Alchemical free energy calculations via metadynamics: Application to the theophylline-RNA aptamer complex*.[22] |
| Differential scanning fluorimetry (DSF) (future perspective) | Has not yet been used with fragments in combination with RNA targets. The method identifies hits through a change in melting temperature of the target molecule when bound to a ligand. | **Silvers *et al*:** *Differential Scanning Fluorimetry for Monitoring RNA Stability*.[23]<br>**Mashalidis *et al*:** *A three-stage biophysical screening cascade for fragment-based drug discovery*.[24] |
| Displacement assays (future perspective) | Has been used for small molecules against RNA targets and for fragments against DNA targets, but not for fragments against RNA targets. The method identifies hits through displacement of fluorescent intercalators. | **Nasiri *et al*:** *Targeting a c-MYC G-quadruplex DNA with a fragment library*.[25] |
| Small molecule microarray (SMM) (future perspective) | Has not yet been used with fragments but has been used against RNA targets. The method utilizes covalently bound molecules on microarrays, providing high-throughput screening capacity. | **Connelly *et al*:** *Synthetic ligands for PreQ1 riboswitches provide structural and mechanistic insights into targeting RNA tertiary structure*.[26] |
| Second-harmonic generation (SHG) (future perspective) | Has not yet been used with fragments in combination with RNA targets. The method identifies hits through a change in intensity of light emission caused by the conformational change of a ligand-bound, dye-labeled target molecule. | **FitzGerald *et al*:** *Discovery of fragments inducing conformational effects in dynamic proteins using a second- harmonic generation biosensor*.[27]<br>**Birman *et al*:** *Second-harmonic generation-based methods to detect and characterize ligand-induced RNA conformational changes*.[28] |
| Cryogenic electron microscopy (Cryo-EM) (future perspective) | Has not yet been used with fragments. Provides structural information on hit-target interaction while requiring less amount of target compared to X-ray crystallography. | **Herrero del Valle *et al*:** *Prospects for antimicrobial development in the cryo-EM era – a focus on the ribosome*.[29] |

## 2. Fragment screening methods used against RNA

The screening methods listed in Table 1 that have recently been applied for FBDD against RNA have been further described below.

### 2.1. RNA observed $^{1}$H-NMR-based fragment screening

Target-observed $^{1}$H NMR fragment screening measures the change in chemical shift and peak intensity in functional groups within the biological target, caused by binding of a ligand which changes the local chemical environment as exemplified in Figure 1B from a study by Lee *et al*.[10,14]

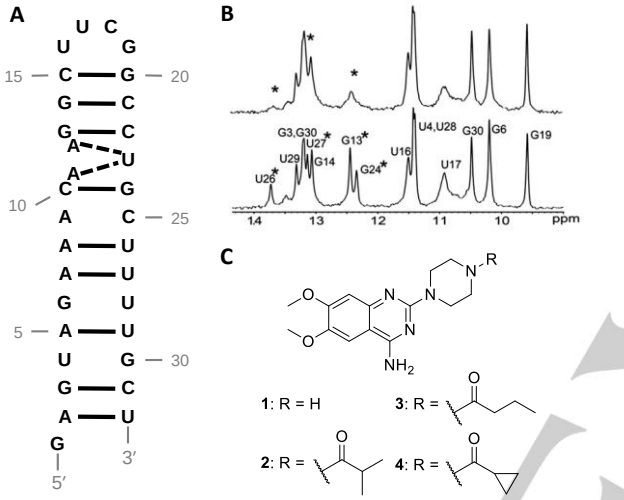


**Figure 1.** (A) The model RNA hairpin of the influenza A virus RNA promoter targeted by Lee *et al*. (B) The $^{1}$H NMR spectra of selected imino protons from the targeted RNA promoter with (below) and without (above) a binding ligand. The specific peaks that change are marked with a star. (C) The structure of the strongest hit (DPQ, **1**) identified by Lee *et al*. and three analogues (**2**, **3**, and **4**) subsequently discovered by the group to show antiviral activity.[14] Figure 1B adapted from Ref. [14] by Lee *et al.* with permission from The Royal Society of Chemistry, copyright 2014.

In this study, the authors used RNA-observed $^{1}$H NMR fragment screening for the influenza A virus RNA promoter, which is a highly conserved sequence among most variants of influenza A virus.[14] A hairpin loop representing this anti-viral RNA target (Figure 1A) was subjected to $^{1}$H NMR-based fragment screening at a concentration of 40 μM, with a library of 3677 fragments. The initial screening used cocktails of 20 fragments, each at a final concentration of 100 μM, to screen multiple fragments at once, increasing throughput. They identified 6 fragments that perturbed the $^{1}$H NMR spectrum in the imino proton region of the RNA target (an example of this is shown in Figure 1B). The authors attributed the low hit rate of 0.2% to the generic nature of the library. Subsequently, the strongest binding fragment hit, 6,7-dimethoxy-2-(1-piperazinyl)-4-quinazolinamine (DPQ, **1**), was modified at the piperazine ring. This approach led to three novel compounds with antiviral activity (**2**, **3**, and **4**), however their selectivity for the influenza virus A RNA promoter remains to be validated.[30]

RNA-observed NMR screening is particularly useful when the target structure is known, as the chemical shift change from binding also provides information about which specific regions in the target interacting with the ligand. However, this method also has limitations: since the conformational change is observed through the NMR signals for the target, it is not possible to determine which of the ligands in the screening cocktail are binding, requiring subsequent screening of the individual fragments of that cocktail, which limits throughput. Additionally, compared to 20 amino acids for proteins, RNA is built from only four different nucleotides, meaning overlap of NMR signals quickly become a challenge as the size of the RNA molecule increases.[31,32] Full or site-specific labeling with $^{13}$C, $^{2}$H, $^{19}$F, or $^{15}$N can alleviate this problem but also shifts the limitation to the demand of a labeled target molecule.[32,33]

### 2.2. Ligand-observed NMR

In ligand-observed NMR changes in the ligand signals in the absence and presence of the target molecule are evaluated. In case of protein targets, this method is found to generally require lower concentrations of the target than in target-observed NMR.[10,33] Additionally, the underlying principle makes the size of the target less critical, negates the need for deconvolution by re-screening cocktails of fragments containing hits, and does not require a known structure of the target. These benefits suit RNA-targeted FBDD programs well, and two ligand-observed NMR methodologies have been employed for FBS of various RNA targets: $^{19}$F-NMR- and $^{1}$H-NMR-based fragment screening.

#### 2.2.1. $^{19}$F-NMR-based fragment screening

$^{19}$F-NMR ligand-observed fragment screening, as the name suggests, observes target-ligand interaction through changes in intensity or chemical shifts of $^{19}$F resonances. Therefore, $^{19}$F-NMR-based fragment screening requires all fragments to contain at least one fluorine atom.[34] However, this limitation comes with the benefit of an increased chemical shift dispersion of about 100 ppm compared to the ~10 ppm range of $^{1}$H NMR as well as a less cluttered spectrum for each fragment.[15,33] As there ideally is only one magnetically equivalent group of fluorine atoms per fragment this generally allows 20–30 fragments in a cocktail, increasing throughput considerably.[33,34] When performing $^{19}$F NMR screening a $T_2$-filter, the CPMG (Carr-Purcell-Meiboom-Gill) NMR experiment, is commonly used.[33]

Garavís *et al.* were the first to employ $^{19}$F-NMR-based fragment screening against an RNA target: a telomeric repeat-containing RNA ($TERRA_{16}$).[35] They screened a 355-membered fragment library by preparing cocktails of 8 fragments, each at a concentration of 50 μM, against two different target concentrations: 0.25 μM and 0.50 μM. The repeat-sequence of the target allows for a much lower concentration as more ligands can bind to the same target, compared to structures with only one binding pocket. The CPMG spectra of each fragment mixture were evaluated in the absence and the presence of the $TERRA_{16}$ target. Fragments binding to the macromolecular target will experience a reduced $T_2$, broadening the resulting $^{19}$F NMR peak and decreasing the intensity as seen in Figure 2. The screening resulted in 20 hits which underwent further hit validation (*vide infra*).

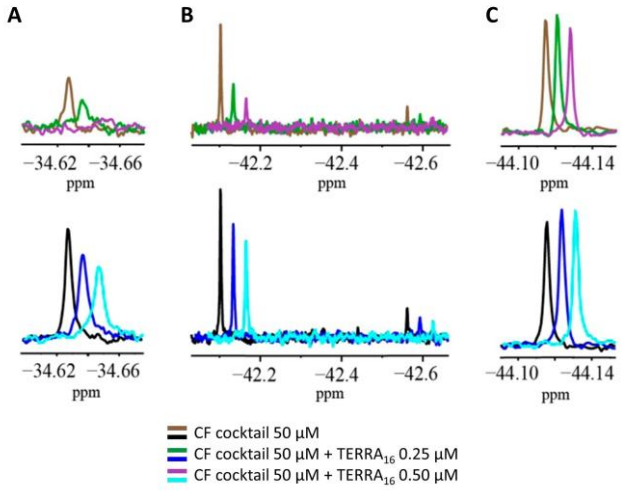


**Figure 2.** $^{19}$F NMR spectra of (A) a hit fragment, (B) both structures of a racemic mixture of a hit fragment, and (C) a non-hitting fragment from the study by Garavís *et al*. Spectra were recorded with a $T_2$ filter (top) and without a $T_2$ filter (bottom).[35] Adapted from Ref. [35] by Garavís *et al.* with permission from American Chemical Society, copyright 2014.

Recently, Binas *et al.* employed $^{19}$F NMR CPMG $T_2$-based fragment screening against 14 different RNA targets alongside five DNA and five protein targets to assess the druggability of RNA.[15] The RNA targets were of different size and architecture, and included small stem loop structures, aptamer domains of riboswitches, full-length riboswitches, terminators and antiterminators of riboswitches, ribozymes, and tRNAs. The DNA targets consisted of both regular double stranded and G-quadruplex DNA structures while the proteins were a combination of RNA-binding proteins, kinases, and phosphatase. In this case the benefits of $^{19}$F NMR fragment screening are more apparent as large cocktails containing 20–21 fragments were used. This allowed them to screen the library of 102 fragments in only five sets of NMR experiments. The screening produced a total of 69 hits – importantly with at least one hit on each target – with riboswitches having the highest hit rates in the range of 7–26% among the RNA targets that otherwise had a hit rate in the range of 1–7%. The authors found that the RNA structures containing loop regions, bulges, and internal loops (like the binding pockets of proteins) had the highest hit rate among the RNA targets. About 20% of the fragments binding to RNA were also found to bind proteins, and proteins did express the highest hit rate (25% on average), but the authors did not find any significant difference between compound classes or physicochemical properties between binders to either RNA, DNA, or proteins.

### 2.2.2. Ligand-observed $^1$H NMR fragment screening

Alternatively, ligand-observed $^1$H NMR fragment screening has also been applied successfully. A main benefit of this method is that there is no requirement for specifically labeled fragment libraries. However, as the method relies on changes in intensity and chemical shift of $^1$H resonances, the signal perturbations are observed for all the protons of each fragment within a cocktail, which limits the typical size of cocktails to around 8–10 fragments.[33,36]

A recent example is from a study where Davidson *et al.* first discovered an arginine-derived chemical probe (arginine 4-methoxy-β-napthylamide) that specifically binds to the tat protein binding region within the trans-activation response (TAR) RNA element from HIV-1 to screen a library of 250 fragments.[37] The probe was used to identify fragments binding at the same site through detection of inter-ligand nuclear Overhauser effect (ILOE) which helped the authors exclude non-specific binders. They also found that the probe locked the target in a specific conformation, enabling screening using 1D saturation-transfer-difference (STD) NMR as well.[37] Using cocktails of 5–8 fragments at 500 µM each and RNA at 20 µM, a total of 20 fragments were identified as primary hits from a library of 250. Later, 6 of these hits were validated leading to a hit rate of 2.4%.

Similarly, Tam *et al*. probed the druggability of the ribosomal peptidyl transferase center (PTC) of *M. tuberculosis* (Mtb) against a library of ~1000 fragments by measuring $^1$H NMR CPMG $T_2$ magnetization decay rates of fragments in the absence and presence of a 29-mer RNA model of the PTC (Figure 3A).[16] They screened the fragment library against the PTC (15 µM) by using a pool of 9–11 fragments, each at a concentration of 300 µM. This screen provided nine hits, giving a hit-rate of 0.9%. The authors found an abundance of phenyl thiazole scaffolds among the hit fragments (Figure 3B), and decided to further screen phenyl thiazole moiety-containing compounds by virtual screening (*vide infra*), resulting in the identification of two lead compounds with potential anti-tuberculosis activity (Figure 3C).

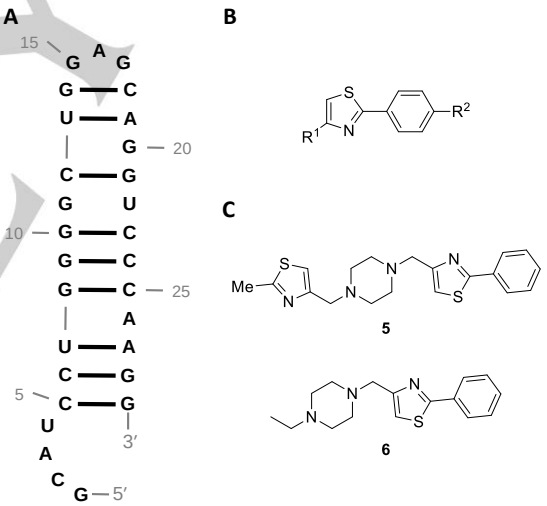


**Figure 3.** (A) The model hairpin 91 of the ribosomal PTC of Mtb target by Tam *et al*. using ligand-observed $^1$H NMR fragment screening. (B) The phenyl thiazole scaffold found to be abundant in the fragment hits. (C) The two lead compounds developed based on the fragment hits of the $^1$H NMR screening.[16]

### 2.3. Labeled ligand displacement methods

Ligand displacement methods rely on the ability of true binders to displace known ligands from the target. To probe the displacement events, the common practice is to label the known ligands with a fluorophore or radioisotope. Fluorescence-based screening methods are of particular interest due to their sensitivity to changes in the proximal chemical milieu and convenient adaptability to a microwell plate-based format for

faster screening at relatively reduced cost. While various fluorescence-based methods have been successfully used against a range of RNA targets, applying them to fragment screening is challenging due to the lower affinity of fragments.[10,11] Zeiger *et al*. adopted a fluorescence-based competition assay,[17] originally developed to screen tripeptides against the HIV-1 TAR RNA sequence,[38] to screen a 29-membered fragment library in a 96-well plate format. The assay tests for the fragments' ability to displace a Tat peptide ligand labeled with fluorescein and rhodamine, which in turn leads to a decrease in the fluorescence as the peptide folds up on itself, causing contact quenching of the FRET signal (Figure 4).

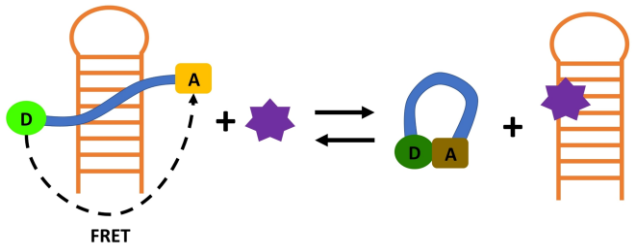


**Figure 4.** The principle of the fluorescence-based competition assay. A peptide (blue) with two terminal dyes (a donor, green and an acceptor, yellow) is stretched out when bound to the RNA target (orange). This holds the dyes within range for FRET causing a strong emission. If a fragment (purple) binds the RNA competitively, the peptide is released, allowing it to fold up on itself and causing the dyes to dimerize. This causes intramolecular contact quenching of FRET, resulting in weak emission.[38]

The authors demonstrated the suitability of the assay to identify binders with $IC_{50}$ values in the range of 20 mM–40 μM. Two promising fragments were discovered (Figure 5C), with one of them (**7**) being a known Tat-TAR inhibitor. While this method is clearly applicable to FBDD, it requires the binding of a peptide to the RNA target, which limits general applicability.

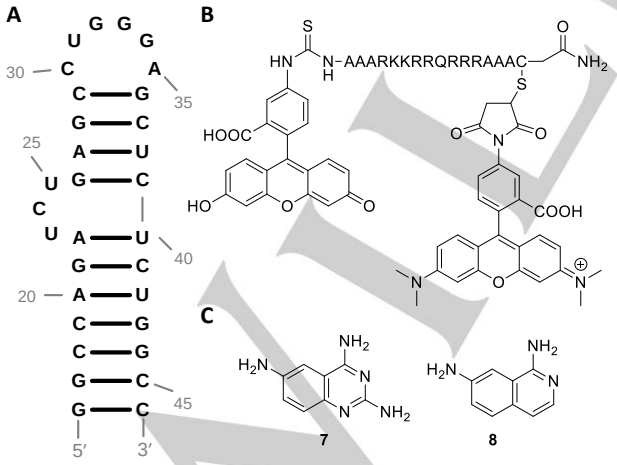


**Figure 5.** (A) The model RNA hairpin of the HIV-1 TAR. (B) The Tat peptide model with the fluorescent dyes fluorescein and tetramethylrhodamine (left and right respectively). (C) The two hit fragments selected by Zeiger *et al*. for further development.[17]

Another example of using a displacement assay for fragment screening involves radioisotope labeling. The TPP riboswitch is one of the promising targets for novel antibiotics,[7] and in order to explore its druggability Cressina *et al*. applied a fragment-based approach.[18] They screened a 1300-membered fragment library against the TPP riboswitch based on the percentage displacement of a cognate thiamine ligand radiolabeled with $^{3}H$. Determination of affinity of the resultant 20 hits by isothermal titration calorimetry (ITC) reduced the list to 17. The screening was performed in cocktails of five but the need of radiolabeling could limit its popularity for fragment screening. Lastly, as the ligand for an RNA-target is not always known, the scope of labeled ligand displacement-based methods for fragment screening can be considered somewhat limited.

### 2.4. Chem-CLIP-Frag-Map

Suresh *et al.* have recently published a method using chemical cross-linking and isolation by pull-down (Chem-CLIP) for fragment mapping (Frag-Map) against a 5'-$^{32}$P-labeled pre-miR-21 RNA, a non-coding RNA linked to cancer.[19] Their method used a previously developed library of "fully functionalized fragments" (FFFs).[39] Each fragment contained a photoaffinity group (here diazirine, see Figure 6) in order to covalently capture the fragments that bind the target. Furthermore, each fragment contained an alkyne group which can react with biotin azide via click chemistry. The latter enables the fragment-bound RNA to be isolated using streptavidin-coated magnetic beads after which binding can be quantified by radioactivity. Finally, the binding site is mapped by reverse transcription followed by dideoxy sequencing.

The group screened 460 FFFs and identified 21 hits resulting in a hit rate of 4.5%. To explore the utility of this approach in FBDD, hit FFFs that did not bind to the same site as pre-miR-21 were further determined through competitive (C-)Chem-CLIP using TGP-21, a previously identified ligand of the Dicer site in pre-miR-21.[40,41] The resultant hit FFFs were fused with TGP-21 to obtain a novel bioactive compound with increased potency as well as selectivity. As stated, the method requires all members of the fragment library to have specialized functionalities which can be a limitation regarding library design.

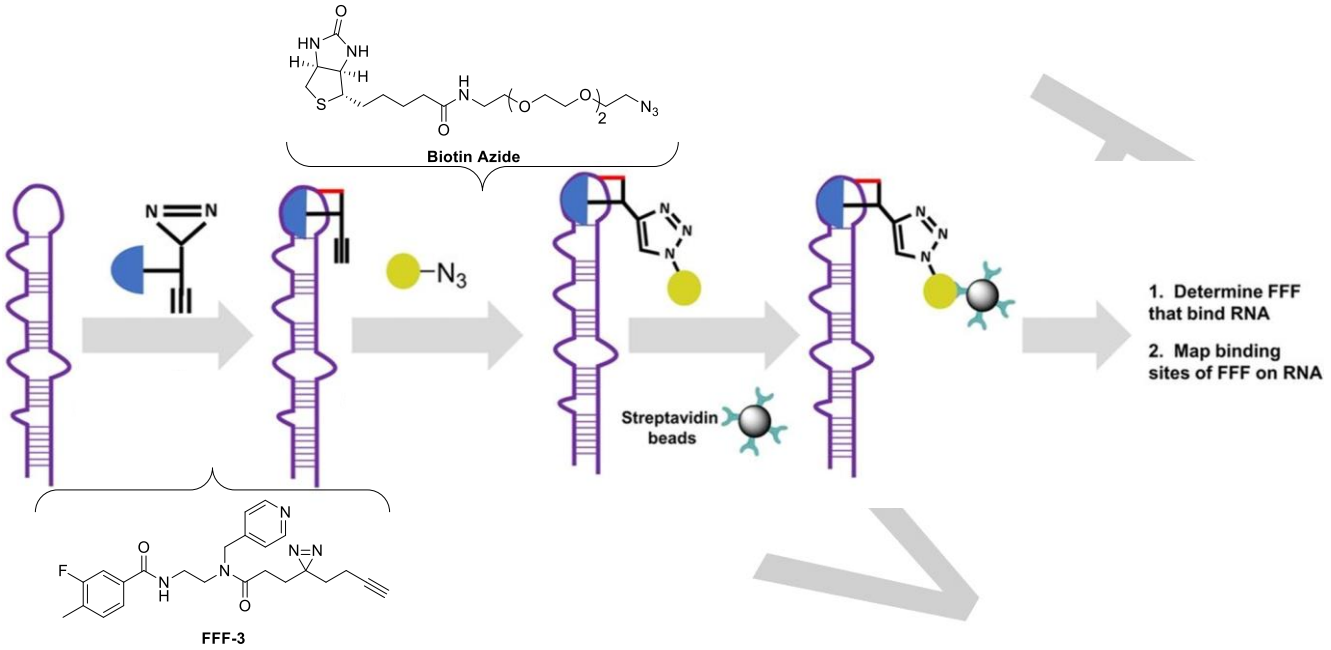


**Figure 6.** Binding of a diazirine and alkyne labelled fragment to an RNA target which is then cross-linked through UV radiation. Biotin azide is attached through click chemistry and the structure is isolated using magnetic streptavidin-covered beads.[19] Adapted from Ref. [19] by Suresh *et al*.

### 2.1. Virtual screening

As far as we are aware, there is only one example in the literature where fragments were docked into the RNA binding site and the results were confirmed by X-ray crystallography.[20] In this study, a purine riboswitch (Figure 7A) was chosen to validate RNA-ligand docking. The binding site in this riboswitch is rather small and consequently a screening library was assembled to reflect this, filtering for compounds with a heavy atom count of no more than 18, one or two ring systems, at least one hydrogen bond donor and acceptor, and a net charge between –1 and +2. Even though FBS was not the stated goal of the study, these filters resulted in a 2592-member screening library that fits within fragment parameters. Four weak binders were discovered (Figure 7B) and for three of them (**9**, **10**, and **11**) the binding modes were determined, demonstrating that high hit rates can be obtained through fragment-based virtual screening (FBVS).

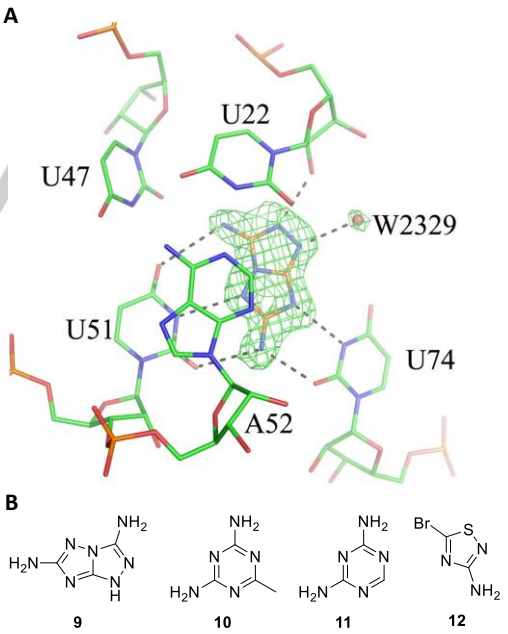


**Figure 7.** (A) The crystal structure of the binding site of the Bacillus subtilis xpt-pbuX guanine riboswitch carrying a C74U mutation (GRA) with the determined binding pose of **9**. (B) The four binders identified by Daldrop *et al*. using RNA-ligand docking FBS.[20] Figure 7A adapted from Ref. [20] by Daldrop *et al.*

## 3. Hit validation and optimization

Hit validation is an important aspect when carrying out FBDD due to the inherently low affinity of fragments. A hit fragment

structure must go through numerous modifications of growth, merging, and/or linking before reaching a lead compound. The entire process could prove very difficult without structural information compared to the chemical elaboration of small molecule hits identified from HTS campaigns.[10] As structural information on RNA currently is more limited as opposed to proteins, methods that identify binding modes are even more critical. While X-ray crystallography and NMR remain the major workhorse for RNA targets with sophisticated 3D structures, biophysical methods such as Selective 2'-hydroxyl acylation analyzed by primer extension (SHAPE) prove critical for RNA targets with less defined structures.

### 3.1. X-ray diffraction

Using X-ray crystallography as a method for fragment hit validation against RNA targets is rare, with Warner *et al.* being the first to apply this method in 2014[21] for validation of fragment hits against *thiM* riboswitch previously identified by the group.[18] They first used X-ray crystallography to determine how the fragments bind to their target and to identify core interactions between fragment hits and the target (Figure 8). The authors considered that the co-crystallization would select for a subset of molecules that achieved complete folding like that of the natural ligand of the TPP riboswitch. To investigate this they applied small-angle X-ray scattering (SAXS), a lower resolution structure-determination method,[42] in order to determine the average global conformation of an RNA-fragment complex. Due to the sugar-phosphate backbone, RNA scatters X-ray more strongly than proteins, and it is considered easier to determine/predict RNA secondary structure, which makes SAXS a useful technique to determine the 3D topological structures of large RNAs without requiring crystallization.[43,44]

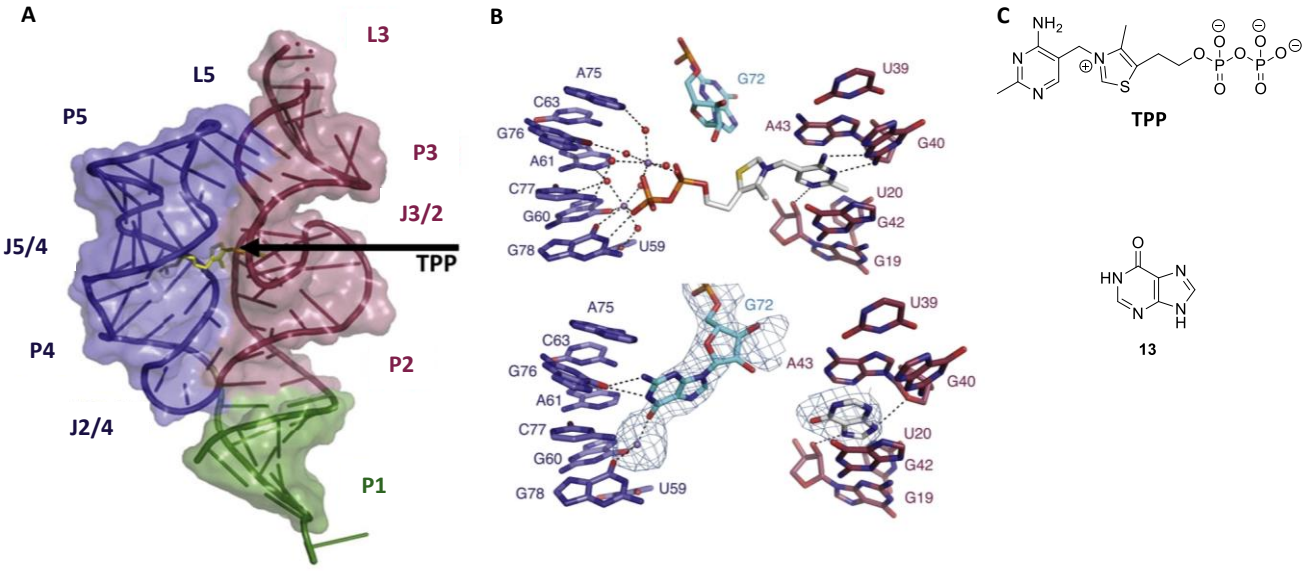


**Figure 8.** (A) A crystal structure of the TPP riboswitch binding the natural ligand TPP. (B) The binding interactions involved in binding TPP (top) and a representative fragment hit (**13**) (bottom) from the Warner *et al*. study. (C) The structures of TPP and fragment **13**.[21] Figures A and B adapted from Ref. [21] by Warner *et al.* with permission from Elsevier, copyright 2014.

Using this method, Warner *et al.* found that the fragment hit induced in solution a compactness of the riboswitch that was intermediate between that of the free and that of TPP-bound riboswitch, despite the co-crystallized structure being highly similar to the TPP-bound one. Further, isothermal titration calorimetry (ITC) was used as an additional hit validation method, as it can be used to measure the binding affinity between ligand and target, including small molecule-RNA interactions.[6]

### 3.2. SHAPE

Selective 2'-hydroxyl acylation analyzed by primer extension (SHAPE) utilizes the difference in nucleophilicity of the ribose 2'-hydroxyl group depending on nucleotide flexibility (Figure 9A).[45] As a ligand binds the RNA, the nucleotide flexibility changes due to formation or breaking of base-pairing, making some nucleotides more prone to acylation than others. Corresponding cDNAs are then produced via primer extension and separated through high-resolution gel electrophoresis. Differences in band intensities between ligand-bound and free RNA show where acylation has increased and provides insight into the secondary structural changes resulting from ligand binding.[45,46] This method was also used in the previously mentioned campaign by Warner *et al.* to validate fragment binding to the RNA target at single-nucleotide resolution.[21] A similar method is the in-line probing assay, which does not require chemical modification but instead relies on an in-line nucleophilic attack on a phosphodiester bond by the 2' oxygen of a spatially adjacent nucleotide to cleave the RNA molecule as seen in Figure 9B.[47] This was used to validate hits in the previously mentioned study by Tam *et al*.[16]

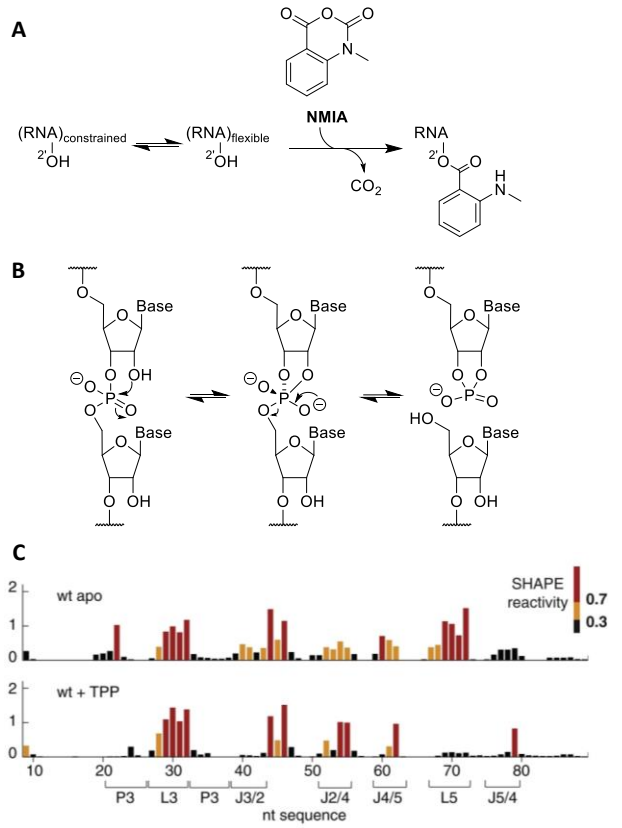


**Figure 9.** (A) The mechanism for selective 2'-hydroxyl acylation of RNA for SHAPE analysis using the N-Methylisatoic Anhydride (NMIA) reagent.[45] (B) The mechanism of the in-line nucleophilic attack of the 2′ oxygen in an RNA phosphodiester promoted by stabilized alignment with the 5′ oxygen–phosphorus bond.[47] (C) SHAPE results from the study by Warner *et al.* showing a change in SHAPE reactivity when the riboswitch binds TPP.[21] Figure 9C adapted from Ref. [21] by Warner *et al.* with permission from Elsevier, copyright 2014.

### 3.3. NMR

Structural analysis by NMR spectroscopy can be a valuable tool to determine the specific interactions between a bound fragment and the RNA target. When combined with RNA-observed $^{1}$H NMR screening as used in the previously mentioned campaign by Lee *et al.*, the same imino peaks used to identify hit compounds can be used to pinpoint fragment-RNA interactions.[14] An analysis of the peak intensities from the $^{1}$H NMR spectra of different concentrations of the hit fragment against the RNA target also allowed the authors to determine the $K_D$ of the strongest binding fragment. Lee *et al.* determined 741 experimental NMR spacial and angular restraints from a range of other homonuclear and heteronuclear 2D experiments. These restraints were then used to calculate the RNA-fragment structure computationally using the structure generation engine X-PLOR.[14,48]

$^{1}$H–$^{15}$N correlated NMR data of $^{15}$N-labeled RNA targets can also provide highly accurate information on fragment-target interactions as demonstrated by Binas *et al.* in their previously mentioned 2020 study.[15] They combined these 2D NMR results with a $^{19}$F NMR competition assay using a known ligand. While this could not provide an exact structure of the ligand-target interaction, they reasoned that an incomplete recovery of the fluorinated ligand upon addition of the known ligand could be due to the fragment binding allosterically, while a closer to complete recovery would signal direct binding. Due to small perturbations of the chemical shifts, they also employed $^{1}$H–$^{1}$H TOCSY experiments where more significant perturbations could be observed for aromatic hydrogen cross peaks of pyrimidine residues.

Zeiger *et al.* also used $^{1}$H–$^{1}$H NOESY experiments in their above mentioned 2014 study to determine and assign the imino region on their RNA fragment structure, combining this with $^{1}$H NMR titration of the target with the hits, to determine which specific nucleotides interacts with the ligands.[17] This allowed them to identify that two of their most potent hits (Figure 5C) bound at neighboring sites on the target, inspiring fragment-linking hit-to-lead development.

As with the general RNA-observed NMR screening procedure, structural analysis can be limited by the size of the target, as a larger target quickly complicates calculations. This was the case for Garavís *et al.* when using RNA-observed $^{1}$H NMR experiments as hit validation following the above-mentioned $^{19}$F NMR screening. The authors circumvented this problem by producing a smaller fragment of the $TERRA_{16}$ structure ($TERRA_2$).[35] They also used saturation transfer difference (STD) experiments of the full target to further validate the identified hits.

### 3.4. Computational methods

While development and execution of biophysical assays can be time consuming and expensive, structure-based virtual screening is potentially a powerful alternative.[49] The molecular docking methods used for this purpose rely on sequential docking of small molecules from a database into binding pockets of structurally characterized biomolecular targets and scoring for their fit. Molecular docking is applied 1) to identify hits among thousands to millions of molecules, and 2) to predict the binding modes of the ligands. Both objectives require score ranking and consequently the success of docking largely relies on what is called the 'scoring function'. While various docking approaches originally developed for protein targets have been applied to RNA targets, often the scoring functions are re-parameterized to score RNA-ligand complexes to take into account the different chemical environment of RNA binding sites compared to protein binding sites.[3] The performance of various RNA-ligand docking programs and other docking programs originally developed for proteins have been reviewed recently.[3] While often not the stated purpose of the study, for a number of targets fragment-sized ligands were discovered.

Virtual screening RNA for fragment hit discovery can be used alone, or in tandem with other screening methods.[3] While the most relevant study pertaining to the standalone application is discussed above, Tam *et al.* employed virtual screening in tandem with $^{1}$H-NMR against ribosomal PTC.[16] After recognizing the phenylthiazole-containing compounds as fragment hits against *Mtb* PTC from $^{1}$H-NMR-based fragment screening, structure-based virtual screening was used for subsequent optimization. They screened 919 phenylthiazole-containing compounds against *S. aureus* PTC (> 90% sequence identity to *Mtb* ortholog) using AutoDock[50] and identified 10 potential hits, 2 of which were then validated as true binders. In a final step, the docking data was used as a basis for machine learning to suggest guidelines on how to further improve the affinities of the compounds. Alternatively, Tanida *et al.* proposed alchemical free energy calculations via metadynamics to

simultaneously predict the binding pose, based on a comprehensive search and, the binding energy.[22] This is important as rational elaboration of fragment hits require accurate predictions of their binding poses. The method relies on exploring the various conformations of the ligand within the RNA binding site by metadynamics and applying cluster analysis to identify possible binding pose(s). Each of these conformations were then subjected to alchemical free energy calculations to obtain binding energies. Using theophylline-RNA aptamer complex as a model, the most favorable predicted binding pose of theophylline was found to be consistent with that of the NMR structure. Importantly, the overall binding free energy approximated well with the experimental value (-8.9 ± 0.41 vs -8.92 kcal/mol).

Altogether, these studies suggest the utility of computational approaches in hit optimization for RNA targeting FBDD programs. Particularly, the Tam *et al*. study explicitly implicates that computational approaches can complement experimental methods and can even guide rational elaboration of hits.

## 4. Library design

At the core of FBDD lies the significant differences between the fragment libraries and those of more classical HTS libraries, especially the smaller size of the molecules and the heavily reduced size of the libraries. Researchers from Astex Pharmaceuticals proposed the rule of threes (RO3) guidelines back in 2003,[51] but other considerations such as three-dimensionality and ease of chemical elaboration of fragment hits are also relevant.[52,53] As fragment hits generally are low-affinity binders they need to be synthetically linked, grown, or merged so it is more efficient when the library consists of molecules with multiple functional groups as growth vectors.[11] The fragments themselves could also be synthesized via “poised scaffolds” that allows for easier synthesis of analogues of hits for further SAR studies.[54]

Another consideration when creating a fragment library concerns the potential targets of that specific library; should the library be target directed or not? Fragments are more general in covering chemical space compared to their larger counterparts and so it could be argued that target-directed fragment libraries are unnecessary.[10] However, the small size of fragment libraries would allow for a more cost-efficient directing towards a specific target class as the amount of fragments to be synthesized will be limited.[37] It would perhaps even be beneficial to have a core, general fragment library, that could subsequently be combined with auxiliary directed libraries, allowing for efficient use against a highly diverse range of targets.

Many RNA structures such as riboswitches and ribosome subunits contain well-defined binding pockets to allow selective interactions with small molecules and fragments.[3,7,55] Further, RNA binding sites are often similar in size and hydrophobicity to druggable protein binding sites and likewise, many RNA-ligands lie in drug-like chemical space.[56] This suggests that in principle general purpose fragment-libraries should deliver sufficient hits for RNA targets, as also seen in the examples discussed above. However, as there are some significant differences between RNA and proteins regarding structural and chemical environment, incorporating privileged scaffolds for RNA-ligands into RNA-targeted libraries could lead to higher hit rates.[55,57]

RNA-targeted libraries for small molecule HTS have been developed, with notable efforts by the Hargrove and Disney groups.[58,59] On the other hand, there have been comparably fewer efforts to develop RNA-targeted fragment libraries, with the only library specifically designed for RNA targets produced by Bodoor *et al*. in 2009.[60] The authors analyzed seven ligand databases for RNA-binding small molecules with $K_D$ values below 50 μM. For chemically similar compounds, the strongest binder was chosen as representative, providing them with a total of 120 small molecule RNA-ligands. 35 physicochemical descriptors were calculated and compared to those calculated for similarly curated protein-binding small molecules, but the authors found no significant differences between the two sets and chose to rely on designing fragments from privileged structures found in the RNA-binding small molecule set. This was achieved by manually fragmenting the structures mainly at points next to rings and subsequent clustering to reject similar fragments resulting in a 114-compound library.

An alternative method for RNA-specific library design is chemoinformatic analysis of RNA-binding ligands with comparison to protein-binding ligands and screening libraries. This can help determine significant physicochemical or structural differences in binders that can make them more selective against RNA-targets. While there have been extensive studies on chemoinformatics of small-molecule libraries against RNA, similar studies for fragment libraries have still not been performed. This may be due to the relatively few examples of FBDD for RNA targets and the resulting dearth of data for comparative studies. However, some of the knowledge obtained about RNA-ligands in general can also be applied to the design of RNA-targeted fragment libraries.

The Hargrove group found that specifically curated or focused small-molecule libraries may be effective for RNA-targeted drug discovery, as they found RNA ligands to have distinct structural and chemical properties from those of protein-binding ligands.[61] In an article from 2020, the group analyzed PDB structures of RNA containing small molecule binders and compared them to small molecule-protein structures in order to determine if there are any privileged ligand-target interactions that can be exploited.[62] The study was limited by the small amount of high-resolution small molecule-RNA complexes present in the PDB database, with only 37 unique ligands targeting 14 different RNA structures. As can be seen in Figure 10, the group found that RNA-binding ligands favor hydrogen bonding and stacking, compared to protein-binding ligands that primarily favor hydrophobic interactions. The majority of the hydrogen bond interactions determined were from aminoglycosides (Figure 10C) so emphasis should be on the higher amount of stacking interactions. That said, their analysis also showed that hydrogen bonding is important for selective RNA recognition with high affinity. To exploit this one could benefit from including fragments with a higher number of hydrogen bond donors/acceptors compared to the general RO3 guidelines, which are, as should be remembered, based on protein screening results. The group also emphasized that sulfur-mediated interactions are underexplored for RNA-targeted drug discovery, both regarding hydrogen bonding interactions and hydrophobic effects, so this should also be considered when designing fragments.

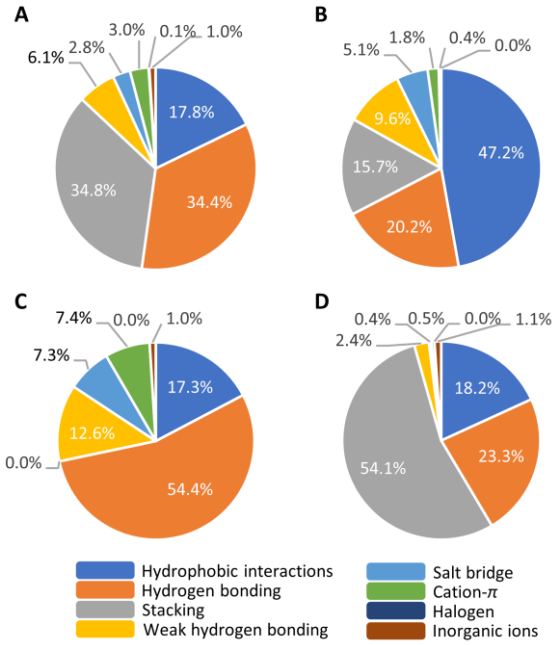


**Figure 10.** Differences in molecular interactions between (A) RNA-binding small molecules, (B) protein-binding small molecules, (C) an aminoglycoside subset of RNA-binding small molecules, and (D) RNA-binding small molecules with dataset C removed.[62]

Regarding the actual structure preferences for RNA-targets, the Hargrove group also performed another study in 2017 where they curated a library of small molecules (excluding aminoglycosides, peptides, and oligonucleotides) binding non-ribosomal RNA, called R-BIND.[58] They then analyzed a range of key physicochemical, structural, and spatial properties and compared these to RNA-binding small molecules from the nucleic acid ligand database (NALDB) and to FDA-approved small molecules, which primarily bind proteins. They found eight physicochemical and structural properties where both RNA-binding ligand libraries deviated significantly from the FDA ligand database: 1) RNA-binders contain more nitrogen atoms, 2) RNA-binders contain fewer oxygen atoms, 3/4/5/6) RNA-binders contain more rings, aromatic rings, heteroaromatic rings, and more ring systems, 7) RNA-binders have lower $Fsp^3$, and 8) RNA binders have fewer stereocenters. They also found that RNA-binding ligands generally are more rod-like shaped.[58,61]

Looking at privileged structures, the Disney group has provided an initial overview on privileged small-molecule scaffolds from where it may be possible to draw inspiration when designing RNA-targeting fragment libraries.[4,59,63] They have determined the benzimidazole scaffold (**14**) as a privileged scaffold and built up a small-molecule library against RNA.[59]

More recently, the Disney group has presented additional potential privileged small-molecule scaffolds based on literature results, consisting of benzimidazoles (**14**), imidazoles (**15**), phenyl imidazolines (**16**), indoles (**17**), N-substituted carbazoles (**18**), alkyl pyridinium scaffolds (**19**), 2-amino-pyridines (**20**), 2-amino-quinolines (**21**), 1,8-diamino-2,7-naphtyridines (**22**), and 2-aminopyrimidines (**23**).[4]

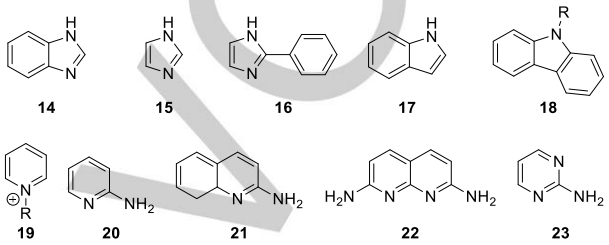


**Figure 11.** Privileged scaffolds collected and presented by the Disney group.[4,59]

In another study by Rizvi *et al.*, 42 diverse RNA targets were screened against about 50,000 drug-like compounds and the results were analyzed to derive properties for RNA ligands.[56] This study suggests that most RNA ligands are in drug-like chemical space, and some privileged scaffolds could be identified among the hits (Figure 12). As one might expect based on the RNA structure and similar to the findings of the Disney group, nitrogen-containing heterocycles were among the most enriched features for RNA binders, while moieties possessing a negative charge (e.g. phosphates and carboxylic acids), were enriched among non-binders. The authors divided the privileged binding scaffolds into two groups depending on whether they bind selectively to a single RNA target and found that many of the highest scoring general binders did not act as selective binders. Interestingly, in contrast to the studies from the Disney group, benzimidazoles were not identified as privileged substructures for RNA-ligands suggesting that privilege might also be RNA class-depending.[56]

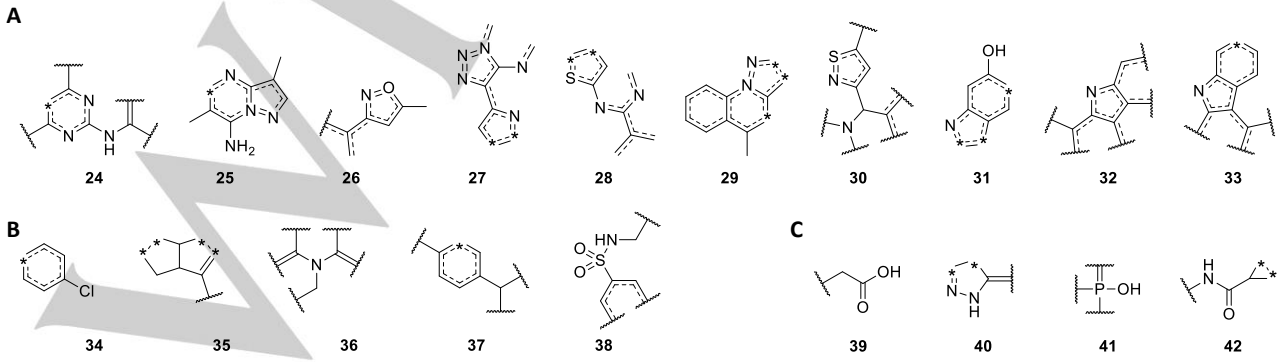


**Figure 12.** Privileged scaffolds identified by Rizvi *et al*. divided into (A) overall RNA-selective scaffolds, (B) specific RNA class-selective scaffolds, and (C) non-RNA binding scaffolds.[56] The stars are heteroatoms.

In addition to privileged scaffolds already presented in the literature, there are also a number of RNA-binding small molecule databases that allow analysis of physicochemical properties, 3D ligand structures, and biological activity.[4]

Inforna is a chemoinformatics tool developed by the Disney group, that can suggest lead-like small molecules based on a given RNA target through comparison of its structural motifs to those known to interact with ligands in their database.[41] The Hargrove group has developed the previously mentioned freely available searchable database called R-BIND of currently 139 non-rRNA biological active binding ligands, that allows the direct search for specific groups of ligands, which is not available using Inforna.[64]

Another database, SMMRNA contains 770 RNA-binding small-molecule ligands which can be sorted by numerous physicochemical descriptors, structure, and RNA-interactions.[65]

The database NALDB contains 3611 small molecules known to bind a wide range of nucleic acids including double stranded RNA, G-quadruplex RNA, and RNA aptamers, hairpins, and bulges, and is searchable by a range of physicochemical descriptors, structure, and nucleic acid interactions.[66]

The privileged small molecule structures and physicochemical properties determined by Rizvi *et al.* and the Disney and Hargrove groups, can be used to design RNA-targeted fragment libraries by acquiring compounds that contain these scaffolds and adhere to the RO3. To increase diversity, other nitrogen-containing heterocycles might be promising. It would be beneficial for the future of FBDD against RNA targets to monitor the success of these libraries and databases to derive more specific recommendations for RNA-directed fragment libraries, maybe even directed to specific RNA classes.

## 5. Future perspectives

As can be concluded from the previous sections, fragment-based screening of RNA targets is developing rapidly, and it is important to get a perspective on what methods and technology will be used in the future. This section will present both new and current technology with a future potential for FBDD for RNA targets.

### 5.1. Differential scanning fluorimetry

Differential scanning fluorimetry (DSF) is widely used as a high-throughput screening method for small molecules against protein targets,[23] utilizing that ligands binding to a protein alters its conformation causing a change in melting temperature which can be studied easily using a specific fluorescent dye.[67] It has more recently also been applied for FBDD against proteins,[10,11] but while DSF against RNA targets has been applied and described previously,[23,46,68] its application for FBDD has not yet been described with success. It was explicitly left out by Mashalidis *et al.* in a 2013 fragment screening procedure protocol when used against riboswitch targets,[24] due to difficulties with calculations of melting temperature, interactions with the fluorescent dye, and inconsistencies in ligand-binding effects on the output.[18]

It seems that DSF has otherwise not been examined regarding RNA-targeted fragment screening, and it is possible that the difficulties encountered by Mashalidis *et al.* are limited to the riboswitch sub-class of RNA targets. It could then be interesting to investigate how this method fared against other RNA-target classes with differences in both size and structure.

### 5.2. Displacement assays

DNA is an alternative nucleotide target and although of course having a different biological role, similar screening techniques can often be applied. One such method could be an intercalator displacement assay (IDA) (or fluorescent intercalator/indicator displacement (FID)) as used by Nasiri *et al.* in 2014[25] in a fragment screening campaign using a 1300-membered fragment library previously used successfully against RNA targets (*vide supra*).[18] The authors targeted the c-MYC G-quadruplex using thiazole orange as the probe, resulting in 10 hits. While the hit rate is low, it is comparable to the 17 hits from their RNA-screening. Grouping the hits based on structural similarity resulted in four fragments having fused 5- and 6-membered heterocyclic rings, two fragments having two fused 6-membered heterocyclic rings, and four fragments being 4-substituted aniline derivatives (Figure 13).[25]

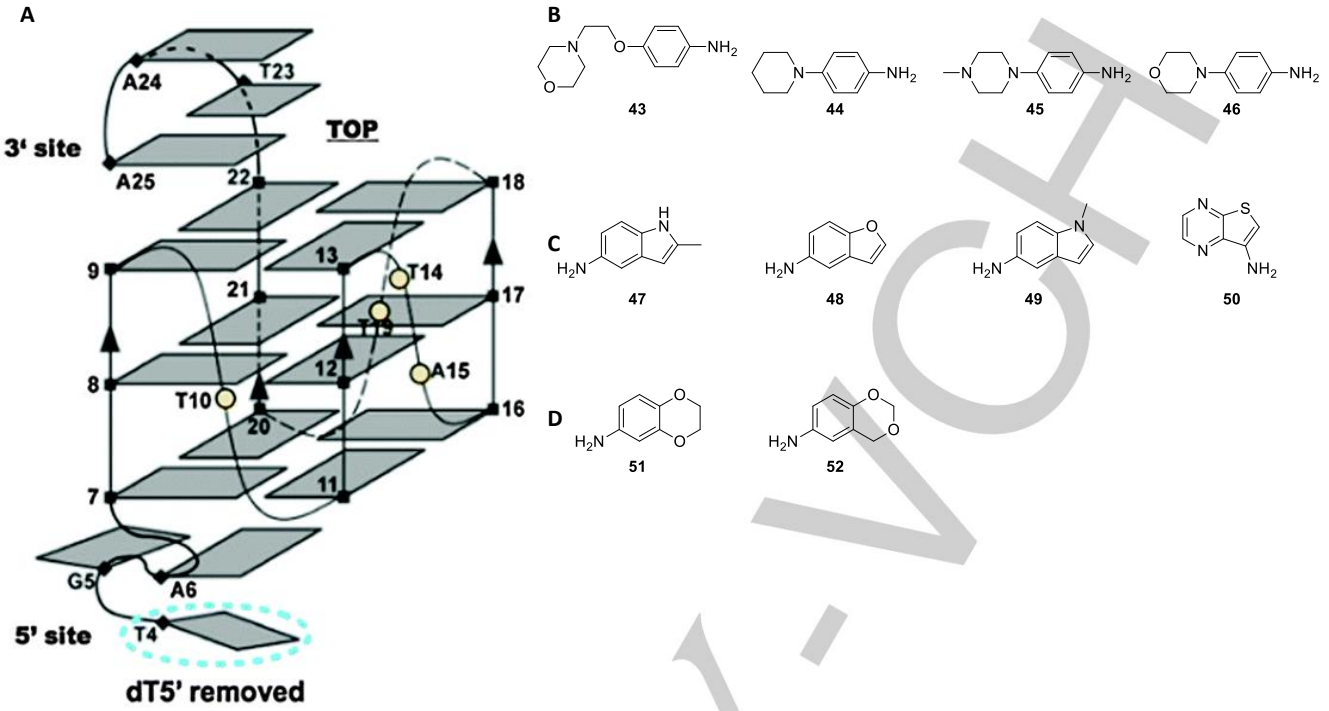


**Figure 13.** (A) A schematic structure of the c-MYC G4 DNA target by Nasiri *et al*. (B, C, and D) The 10 hits from the IDA screening grouped as 4-substituted anilines, fused 5-and 6-membered heterocyclic rings, and fused 6-membered heterocyclic rings, respectively.[25] Figure 13A adapted from Ref. [25] by Nasiri *et al.* with permission from The Royal Society of Chemistry, copyright 2014.

Small-molecule IDA screening against RNA is well described and can be considered as a robust method with high throughput, but since it is a competitive method it requires a known ligand for the target.[6,69,70] As the method has been used successfully for small molecules against RNA targets and for fragments against DNA targets, the prospect of using it for RNA FBDD is promising.

### 5.3. Small-molecule microarrays

Microarrays are two-dimensional arrays with a glass or gold-coated surface where substrates such as nucleotides, proteins, tissues, living cells, and chemical libraries can be immobilized using various methods depending on the substrate.[71,72] Small molecule microarrays (SMMs) is a subclass where small molecules are covalently bound to the array surface, providing a high-throughput vehicle for probing binding for more than 10,000 small molecules on a single slide.[6,72] In a screening campaign against the $PreQ_1$ riboswitch aptamer, Connelly *et al*. screened 26,227 small molecules, affording 243 hits, i.e. a hit rate of 0.93%.[26] Using ligand observed NMR as hit validation and X-ray crystallization for structure-guided development the group identified one hit compound selected for further development (Figure 14). With the hit compound being fragment-like, this approach shows promise for FBDD. However, a limitation is that the fragments must be covalently linked to a chip, limiting the orientations that a fragment can adopt in a binding site and thus potentially negatively affecting the hit rate.

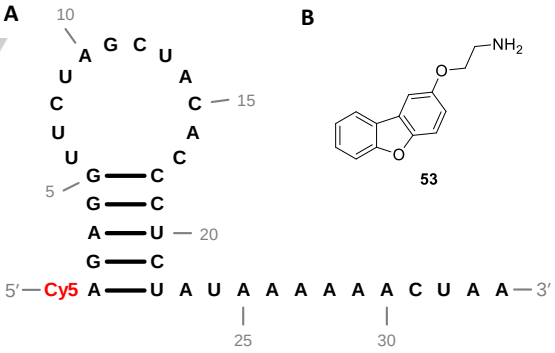


**Figure 14.** (A) The structure of the Cy5-labeled aptamer of the BsPreQ1 riboswitch targeted by Connelly *et al*. (B) The hit molecule identified in the study.[26]

### 5.4. Second-harmonic generation

Second-harmonic generation (SHG) is another biophysical screening method that has recently been used in combination with fragment libraries.[27,73,74] The method requires labeling the target molecule with a dye, immobilizing it, and then radiating it with 800 nm red light (Figure 15). The second-harmonic generation causes two incoming photons to be absorbed by the dye and then emit 400 nm light, and the intensity of the emitted

light is dependent on the position of the dye relative to the surface normal. The method is comparable to SPR, but where SPR only provides binding information such as affinity, SHG provides both binding information and conformational data, making it especially useful for screening of fragments as they have inherently low affinity but can still induce conformational changes upon binding.[73] The method has also been applied with success using small molecules against RNA targets including hairpins and riboswitches by Birman *et al*.[28] The authors optimized the assay for the theophylline aptamer which binds theophylline, a molecule that fits within most of the RO3 fragment guidelines. Even though the group used small molecules to screen against the aptamer, it seems likely that this method could be a valuable tool in FBDD against RNA.

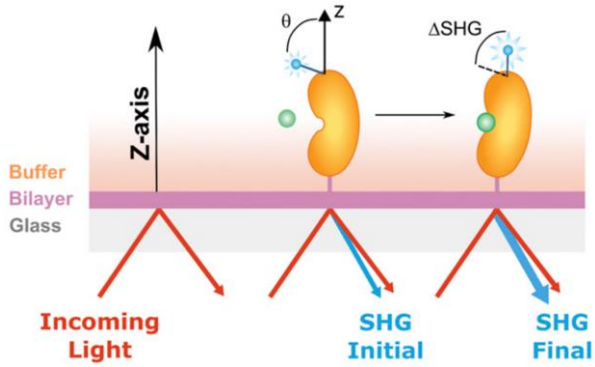


**Figure 15.** SHG screening. An immobilized target with a dye is illuminated with 800 nm red light. Two photons are absorbed and then emitted as one 400 nm blue photon, where the intensity depends on the dye's angle of orientation (θ) relative to the surface normal z.[73] Adapted from Ref. [73] by Donohue *et al.* with permission from National Academy of Sciences, copyright 2019.

### 5.5. Cryogenic electron microscopy

Another promising biophysical screening/hit validation method for fragments against RNA targets is cryogenic electron microscopy (cryo-EM), which can be considered as an alternative to X-ray crystallography. It is a recently improved method and has been used for FBDD against protein targets.[75] Considering RNA as a prospective target class, a perspective by Herrero del Valle *et al.* in 2020 focuses on its use for studying the ribosome.[29] Compared to X-ray crystallography, cryo-EM requires smaller quantities of the target biomolecule and the freezing technique avoids crystallization challenges, making the method available to a larger range of targets.[29,75] The size of possible target molecules also does not play as important a role with size ranges spanning from less than 100 kDa to several MDa.[29]

Herrero del Valle *et al.* found that the resolutions obtained and the throughput rate both indicate that cryo-EM can be a future screening and hit validation method for FBDD with much larger RNA structures such as ribosome subunits being possible targets.[29] They do however mention that there still is a need for further improvement regarding throughput.

### 5.6. X-ray crystallography for primary fragment screening

Throughput improvements have brought X-ray crystallography to the front as a screening method, compared to the use in hit validation mentioned above. Technological advancements have already made X-ray crystallography a viable screening method for protein targets,[76] and it seems likely that X-ray crystallography can also be used as a primary fragment-based screening method against well-behaved RNA structures in the future.

## 6. Summary and outlook

Both FBDD and RNA as a pharmaceutical target are relatively new concepts compared to e.g. HTS against more classical protein targets. It follows that the combination of these two concepts is comparatively less explored with ample room for development. Numerous screening methods already in use for small molecule screening against protein targets are also compatible with FBDD and RNA, but for recent research endeavors NMR (both ligand and target observing) is most popular, often combined with various RNA structure-elucidating methods such as X-ray crystallography, SAXS, SHAPE, and computational methods.

Fragment library design is an important aspect of the FBDD method, but limited information on ligand-RNA interactions complicate efforts to design a library compared to proteins. Recent reports of physicochemical and molecular properties of small molecule RNA-binders as those by Rizvi *et al.* and the Hargrove and Disney groups could lead to more RNA-focused fragment libraries in the future, but there is clearly a need to obtain more information on the performance of fragment libraries against RNA. Additional screening and validation methods may be further developed for FBDD against RNA targets in the future, such as DSF, SHG and cryo-EM. Improvements in computational chemistry and increased data on RNA structures combined with the use of machine learning tools may also help pave the way for more efficiently targeting RNA in drug discovery.

## Acknowledgements

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## Entry for the Table of Contents

A new exciting frontier for fragment-based drug development is the targeting of RNA structures, as these have been generally underexplored compared to proteins. A review of current and future screening methods, hit validation and optimization, and library design will help facilitate development within this field.