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SURVEY AND SUMMARY

Insights into the development of chemical probes for RNA

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ABSTRACT

Over the past decade, the RNA revolution has revealed thousands of non-coding RNAs that are essential for cellular regulation and are misregulated in disease. While the development of methods and tools to study these RNAs has been challenging, the power and promise of small molecule chemical probes is increasingly recognized. To harness existing knowledge, we compiled a list of 116 ligands with reported activity against RNA targets in biological systems (R-BIND). In this survey, we examine the RNA targets, design and discovery strategies, and chemical probe characterization techniques of these ligands. We discuss the applicability of current tools to identify and evaluate RNA-targeted chemical probes, suggest criteria to assess the quality of RNA chemical probes and targets, and propose areas where new tools are particularly needed. We anticipate that this knowledge will expedite the discovery of RNA-targeted ligands and the next phase of the RNA revolution.

INTRODUCTION

The field of RNA biology has exploded in recent years with the discovery of non-coding RNAs (ncRNAs) that regulate essential processes in all living organisms (1). These processes include transcription, translation, and evasion in bacteria and archaea (1,2) as well as replication, persistence, and cellular transformation in viruses (3). Within the human genome, protein-coding genes are vastly outnumbered by regulatory ncRNAs that can influence a wide range of cellular functions (2,4). Many of these ncRNAs are dysregulated in and implicated as drivers of various human diseases, including metastatic cancers and neurological and neuromuscular disorders (2,5,6). This 'RNA revolution' is radically changing our understanding of the role RNA plays in fundamental biology and is rapidly driving scientific innovation.

Methods and tools to structurally and functionally characterize RNAs at the molecular level, however, are more difficult and/or lacking as compared to those for proteins (7–10). One important example is the development of chemical probes, which has greatly progressed the study of proteins and related diseases (11,12) but has been challenging for non-ribosomal RNAs. This powerful chemical tool requires small molecules with well-defined biological activity, cell permeability, and selectivity to accurately and reliably probe specific mechanistic and phenotypic questions (11,12). Given the potential advantages of small molecule chemical probes over biological approaches (e.g. siRNAs, ASOs and CRISPR-Cas) (13,14) and the power of using both approaches in tandem (12), the development of RNA-targeted chemical probes has the potential to greatly benefit both chemists and biologists interested in RNA.

While ligands that bind non-ribosomal RNA *in vitro* have been reported for decades, the development of chemical probes with evidence of specific small molecule:RNA engagement in cell or animal models has dramatically increased in the last four years. Recent studies report several drug-like small molecules that target a range of RNAs in animal models, including riboswitches (15), miRNAs, (16,17) splice sites (18), and mature mRNAs (19), at least one of which is currently in clinical trials (NCT02268552). Multivalent ligands have been reported that target r(CUG)^{exp} repeats of Myotonic Dystrophy Type 1 (DM1) in *D. melanogaster* (20) and mouse models (21). These recent successes confirm that selective RNA targeting is achievable in biological systems; however, the limited examples over years of effort highlight the challenges associated with selectively probing RNA.

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Recently, we compiled the RNA-targeted BIoactive ligaNd Database (R-BIND), which comprised organic small molecules that target non-ribosomal RNAs and show activity in cell culture or animal models (22). For this Survey, the compilation was updated to include chemical probe discoveries through May 2017 for a total of 116 chemical probes (see Supplementary Material, R-BIND_1-1.xls). Aminoglycosides are excluded due to established non-specific binding behavior (23–25) as well as peptides and oligonucleotides due to distinctive medicinal chemistry properties (26,27). The chemical probes were divided into two classes: monovalent, traditional drug-like small molecules (SM) and multivalent ligands (MV) with alkyl, aryl, or peptidyl linkers between multiple binding moieties. Previous work compared the physicochemical, structural, and spatial properties of the small molecules to FDA-approved drugs as well as RNA-binding ligands without reported biological activity (22). This analysis revealed several key differences between these libraries that can in turn be used to bias small molecules toward biological RNA targeting. In addition to that work, the curation of this collection allowed us to gain insights into: (i) the RNA elements targeted; (ii) the design and discovery strategies utilized and (iii) the *in cellulo* characterization of these chemical probes. Herein, we discuss these insights, highlight unique examples, and consider the need to establish standards for cell-based selectivity. We conclude by proposing future directions that utilize our current and prospective chemical biology toolbox to expedite the discovery of chemical probes for RNA.

RNAS TARGETED BY CHEMICAL PROBES

The 116 chemical probes targeted 33 distinct RNA elements, including those from bacterial, fungal, human, or viral systems [Figure 1]. Although some overlap between targets was observed, the small molecules probed a wider range of RNAs in cell culture than the multivalent ligands [Figure 1A]. The most common small molecule target was the HIV-1 Trans-activation response element (TAR) RNA, a well-studied and frequently screened RNA that binds to the viral protein Tat (23). Disruption of this interaction reduces viral production and represents an alternative strategy against HIV. Some of the first RNA-targeted chemical probes were developed for TAR RNA, including a tetraaminoquinozaline (28) and a 6-aminoquinolone (29,30) with an EC_{50} value of 16 μM and an IC_{50} value of 0.85 μM, respectively, in chronically infected HIV cell models. On the other hand, only one bioactive small molecule was identified for a fungal target, specifically the *Candida albicans* LSU Group 1 Ribozyme (31). This essential ribozyme is a desirable antifungal target as it leads to failed ribosomal assembly when mutated and is absent in the human genome. Further, 10/75 small molecule chemical probes demonstrated efficacy in animal models, targeting seven unique RNA elements in bacterial and human systems. One recent example (19) targeted the G-quadruplex structure located in the 5′-untranslated region (UTR) of Human Vascular Endothelial Growth Factor (*hVEGF*) mRNA, an angiogenic growth factor involved in tumor progression. In a breast cancer mouse model, the small molecule showed antitumor efficacy similar to that of doxorubicin but with fewer indications of side effects.

Multivalent chemical probes targeted fewer distinct RNA elements, with 27/41 unique ligands targeting nucleotide repeat expansions [Figure 1B]. There are several advantages to targeting these RNA repeats: (i) long repeat stretches are typically not present elsewhere in the human genome; (ii) nuclear localization minimizes competition with ribosomal RNA and (iii) targetable motifs are separated by a specific distance (32). Another target of interest was the heat shock response element of the σ^{32} factor mRNA in *E. coli.* This RNA element contains a rare, perfectly paired three-way junction that can be stabilized by symmetrical triptycene-based molecules, forming a distinct shape-selective fit (33). This stabilization resulted in an ∼60% reduction in translation of an σ^{32}–GFP fusion protein and could potentially lead to antimicrobial activity (34). In addition, 7/41 chemical probes showed efficacy in animal models, targeting two RNA elements: $r(CUG)^{exp}$ repeats and pri-miRNA-96. Pri-miRNA-96 is an oncogenic RNA that suppresses the translation of a pro-apoptotic protein, FOXO1. In a mouse model of triple negative breast cancer, a modular ligand designed to target the Drosha processing site on the RNA led to a statistically significant reduction in tumor size and to changes in RNA and protein levels consistent with the proposed mode of action (17). Notably, examination of this list further exposes the RNA-driven processes and diseases that still lack functional chemical probes. Ideal RNA targets have defined functional sites and/or clear phenotypes while also being of high abundance, and several untargeted RNAs, ranging from archaeal ncRNA to oncogenic lncRNAs, meet these criteria.

DISCOVERY AND DESIGN OF RNA-TARGETED SMALL MOLECULE CHEMICAL PROBES

Many of the monovalent ligands were discovered through traditional screening methods. Approximately one-third of the RNA:ligand interactions were identified by each of the following approaches: focused-screening (FcS), high-throughput screening (HTS), and HTS followed by lead optimization (HTS-LO) [Figure 2A]. In this Survey, FcS is defined by the use of biased libraries, which are typically based on prior knowledge of a particular chemotype binding to an RNA element. In contrast to FcS libraries, molecules specifically designed to explore structure-activity relationships were classified as lead optimization (LO). The starting points for several of the FcS libraries and/or other small molecule identification strategies included RNA-binding natural products, chemical similarity searching, and scaffold-based synthesis [Figure 2B–D]. We caution that the relative success of these various approaches cannot be evaluated since failed attempts are not typically documented in the literature.

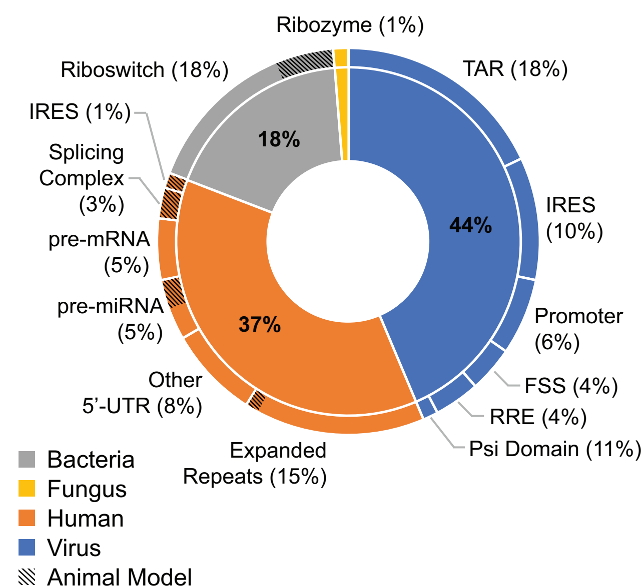
Hit rates

Hit rates are one of the benchmarks used to assess the efficiency of a screen. We note prior to the discussion that comparisons across studies should be interpreted with caution as the definition of a small molecule lead, the specific assays used in primary screens, the controls utilized in

A Small Molecules (SM)

System	RNA Class	RNA Subclass	RNA:SM Interactions*
Bacteria (n = 14)	mRNA	Riboswitch	14 (4)
Fungus (n = 1)	rRNA	Ribozyme	1
Human (n = 29)	miRNA	pre-miRNA	4 (2)
	mRNA	Expanded Repeats	12 (1)
		IRES	1 (1)
		Other 5' UTR	6
		pre-mRNA	4
		Splicing Complex	2 (2)
Virus (n = 34)	mRNA	Frameshift Site	3
		IRES	8
		Psi Domain	1
		RRE	3
		TAR	14
	vRNA	Promoter	5

*Value in parentheses indicates number of RNA:ligand interactions tested in animal models



B Multivalent Ligands (MV)

System	RNA Class	RNA Subclass	RNA:MV Interactions*
Bacteria (n = 2)	mRNA	HSR Element	2
Human (n = 29)	miRNA	pri-miRNA	1 (1)
	mRNA	Expanded Repeats	28 (6)
Virus (n = 11)	mRNA	Frameshift Site	7
		TAR	4

*Value in parentheses indicates number of RNA:ligand interactions tested in animal models

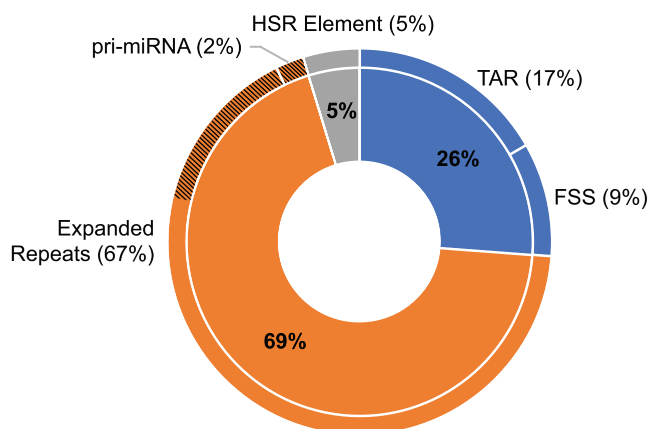
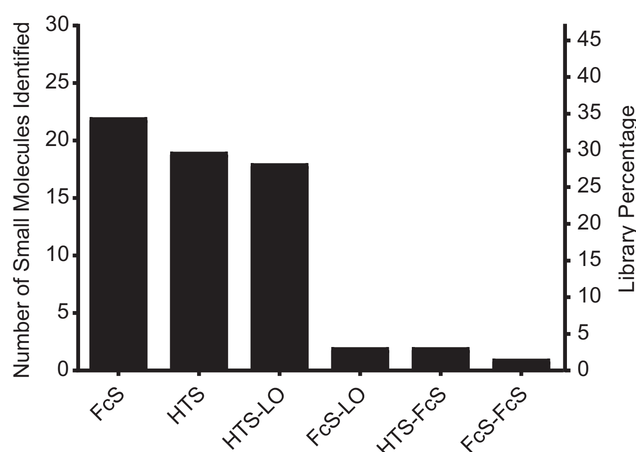


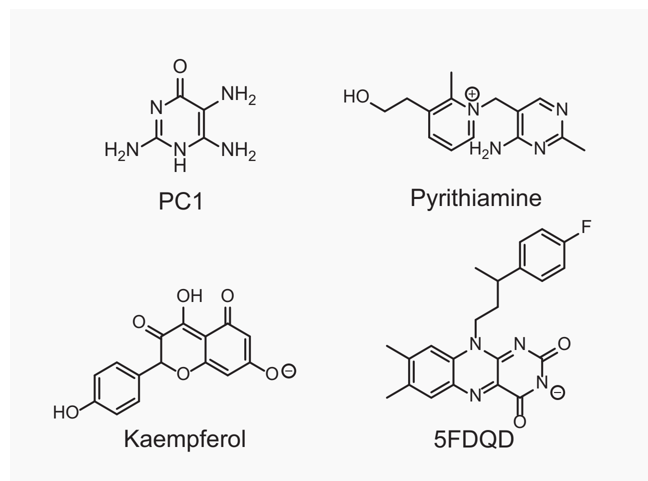
Figure 1. Diversity of RNA elements targeted in living systems. (**A**) The distribution of RNA targets for small molecules (SM): 78 RNA:SM interactions; 75 chemical probes; 33 RNA elements. Of the RNA:small molecule interactions observed, only 10 were tested in animal models for either human or bacterial targets. Despite the large portion of chemical probes for viral RNA targets, none were reported as successful in animal models. (**B**) The distribution of RNA targets for multivalent ligands (MV): 42 RNA:MV interactions; 41 chemical probes; 8 RNA elements. Seven RNA:ligand interactions were tested in animal models. Expanded in R-BIND_1-1.xls. FSS: Frameshift Site, HSR: Heat Shock Response, IRES: Internal Ribosome Entry Site, RRE: Rev Response Element, and vRNA: viral RNA (genome).

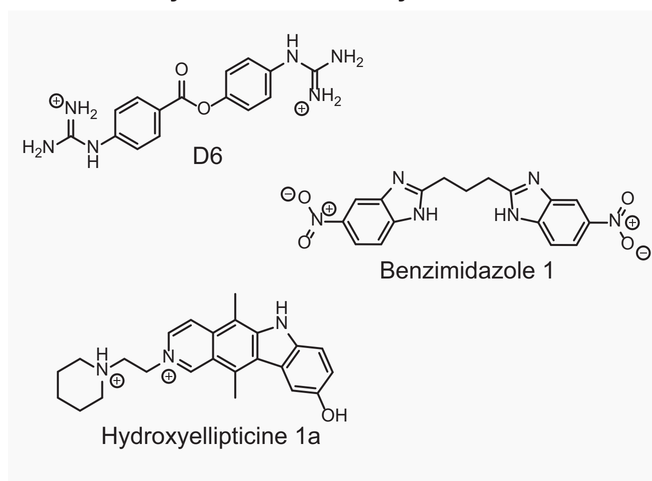
the assay, and the number of false-positives and -negatives can be highly variable and are not always reported. Of the 41 RNA:small molecule interactions discovered through HTS and FcS, 20 had reported hit rates, which were compared by screening approach, primary screen, and primary library [Figure 3]. The higher hit rates found in some FcS approaches provide compelling evidence that FcS is efficient for RNA targets, as it is known to be for protein targets (35,36). Moving forward, characterization of additional RNA tertiary structures (8,9,37,38) and the identification of novel RNA-binding chemotypes (8,39,40) will expedite the FcS approach for discovering biologically active RNA-targeted ligands. While hit rates varied widely within each type of primary screen and primary library, these comparisons support the potential of many distinct paths toward RNA ligand discovery. Specific aspects of library and screen design are discussed below.

Screening libraries

Small molecules discovered by HTS were typically from large libraries ($n > 50\,000$ small molecules), including three corporate libraries and the NIH Small Molecule Repository [Table 1]. There were select examples of smaller libraries as well: FDA-approved drugs ($n = 1120$) and UCLA academic library ($n = 1692$). Importantly, some of these reports explicitly stated that libraries were filtered to yield small molecules with favorable medicinal chemistry proper-







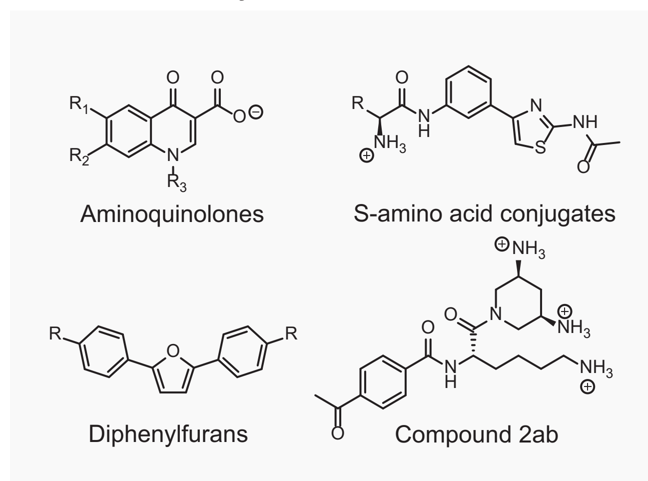


Figure 2. Strategies to discover RNA-targeted chemical probes. (**A**) Focused screens (FcS) identified the greatest number of bioactive molecules ($n = 22$), followed by high throughput screening (HTS) approaches ($n = 19$). Lead optimization (LO) or combined screening tactics have been employed to enhance the activity or pharmacokinetic properties of initial hits from both methods. Library members from FcS and other discovery approaches have been: (**B**) derived from natural products; (**C**) identified through chemical similarity search and/or (**D**) built around synthetic scaffolds. Expanded in R-BIND_1-1.xls.

ties prior to screening. While successful in protein-targeted drug discovery (41), only one report identified a bioactive ligand from a fragment-based library (commercial library) (42). Once optimized, the scaffold yielded four additional molecules that targeted the Influenza A RNA promoter with IC_{50} values ranging from 34 to 44 μM in a cell-based luciferase assay (43). Similarly, only two reports yielded bioactive small molecules from natural product-based libraries (synthetic library and academic library) (19,44). Both of these screens contained fewer than 150 small molecules, yet identified ligands that bind and modulate G-quadruplex structures located in the 5′-UTR of two distinct mRNAs. It is promising that small molecules were discovered from a variety of HTS libraries, suggesting that biologically active, RNA-binding ligands *can* be found in a subset of current small molecule chemical space (22). Further validation and exploration of this space could lead to greater efficiency and success in identifying bioactive leads as well as RNA-privileged chemotypes.

As expected, FcS used smaller libraries, typically containing fewer than 150 small molecules [Table 1]. The largest FcS library ($n = 320$, academic library) was designed through a chemical similarity search of the *bis*-benzimidazole and similar cores, which have shown preferences for 1 × 1 nucleotide internal loops (45). In addition, this library was filtered for favorable medicinal chemistry properties, and the screen resulted in three leads that: (i) bound r(CUG)^{exp} *in vitro*; (ii) led to a statistically significant decrease in *cTNT* mini-gene exon inclusion in cells and (iii) were selective for a mini-gene with 960 r(CUG) repeats compared to a mini-gene without the repeats. FcS also encompassed RNA structure-guided design, which included two studies utilizing molecular modeling to identify ligands structurally similar to guanine for the

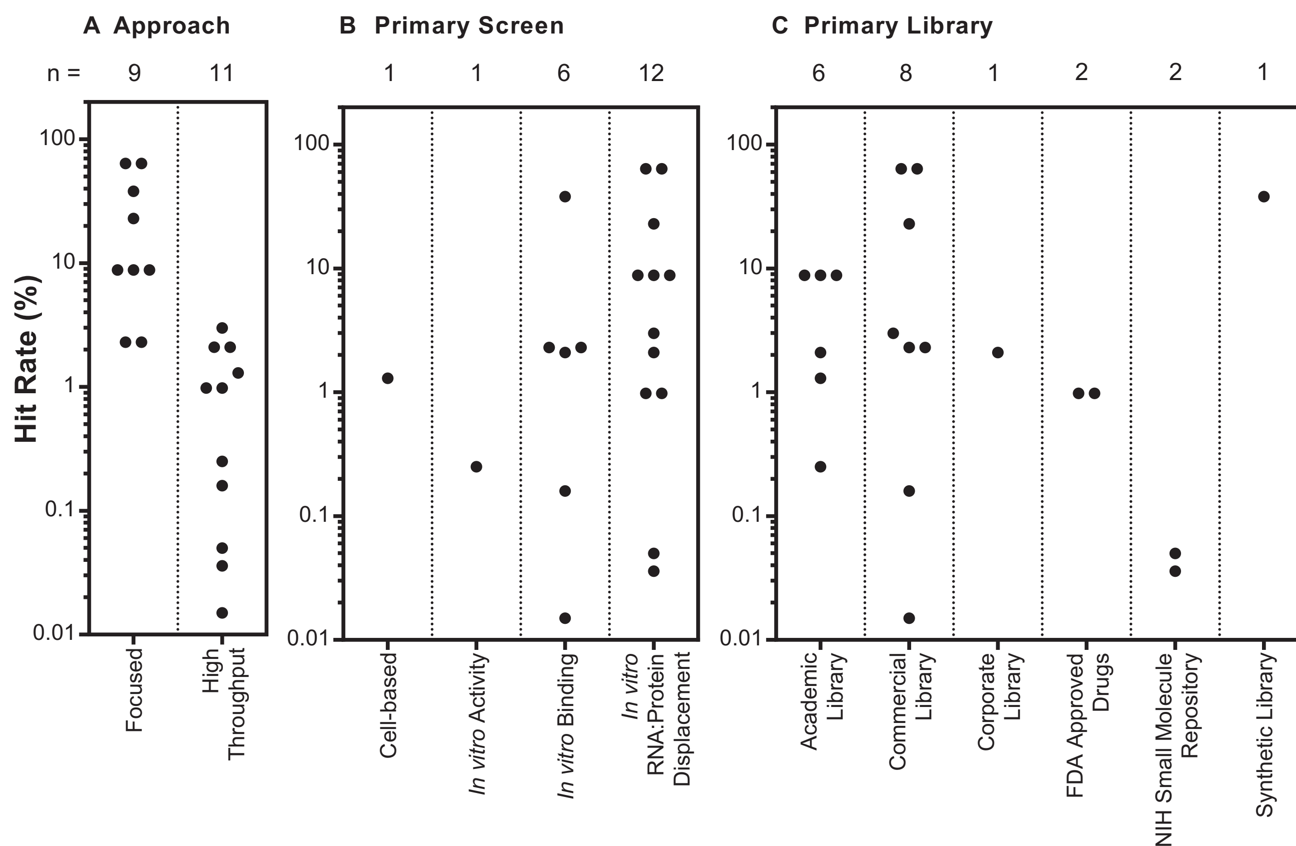


Figure 3. Reported screening hit rates (n = 20) for small molecule chemical probes. (**A**) Hit rates separated by focused or high throughput screening approaches. In general, focused screens resulted in higher reported hit rates. (B, C) Hit rates separated by (**B**) primary screen and (**C**) primary library. Each dot represents a single small molecule. Only examples with explicitly reported hit rates were recorded.

xpt-pbuX riboswitch (46,47). In another structure-guided approach, a small library of *p*-terphenylene-based ligands was designed to mimic an α-helix of Rev, a protein-binding partner of the HIV-1 Rev Response Element (RRE) (48). Leads were selected by docking, with one ligand disrupting the Rev-RRE interaction *in vitro* (IC_{50} = 6.8 μM), inhibiting HIV-1 replication (IC_{50} values of 3.4–5.9 μM), and exhibiting on-target effects via a RRE-luciferase reporter assay (IC_{50} values of 10–21 μM). Further, we did not identify successful reports of biologically active ligands from small molecule libraries biased to general RNA-binding. Recently, chemical companies have designed such focused libraries (ChemDiv, Nucleic Acid Ligands: http://www.chemdiv.com/nucleic-acid-ligands/; Otava Chemicals, RNA Targeted Library: http://www.otavachemicals.com/products/targeted-libraries-and-focused-libraries/rna-binding); however, the success of these libraries is yet to be reported. To date, successful FcS strategies have utilized knowledge of the RNA structure and/or a small molecule binder(s), neither of which is known for many therapeutically-relevant RNAs.

In vitro primary screening assays

The primary screening assay for each small molecule was categorized as computational, *in vitro*, or cell-based [Figure 4A]. This list contained a wide range of primary screening assays, with limited examples of the same assay being used for multiple targets. The majority of the chemical probes were discovered by *in vitro* primary screening assays (n = 28) with fewer in cellulo or silico examples. Of those *in vitro* primary screens, 15 were RNA:protein displacement assays [Figure 4B]. These included fluorescence-based assays (Förster resonance energy transfer (FRET) and fluorescence anisotropy) and radiolabel-based methods (mobility shift, scintillation proximity, and filtration assays). One rather unique assay utilized a molecular beacon approach to probe for stabilization of Stem Loop 3 (SL3), a presumptive structural switch located in the Ψ-Packing Domain in HIV-1 that is destabilized by binding of the Gag protein prior to packaging of the virus (49). In this assay, the 5′- and 3′-terminal ends of the SL3 RNA were labeled with a TET fluorophore and a blackhole quencher (BHQ1), respectively. In the presence of Gag protein, the RNA construct became single stranded and the fluorescence was ‘turned on’. When a small molecule stabilized the folded hairpin form of SL3 RNA, the Gag-promoted RNA destabilization was reduced and the fluorescence was quenched. The researchers screened a modest sized library (>2500 small molecules) and discovered a ligand that reduced viral production similar to models with a mutated Ψ-Packing Domain ($p24_{50}$ = 11.3 μM).

The remaining *in vitro* assays consisted of two activity-based screens and various RNA binding assays [Figure

Table 1. Primary screen libraries of RNA-targeted chemical probes (expanded in R-BIND_1-1.xls)

Type	Source	Description	Library size	SM ligands	Reference
Academic	School of Pharmaceutical Sciences, Sun Yat-sen University	Natural products and related derivatives with diverse structures	144	1	(19)
	Scripps Research Institute	Chemical similarity search of bis-benzimidazole and similar cores and refined for 'drug-likeness'	320	3	(45)
	UCLA Chemical Library	–	1692	1	(53)
	In-House Library	Filtered using Lipinski's and Veber's rules and selected based on chemical structure diversity	8000	1	(50)
Commercial	NR	Chemical similarity search of RNA-binding ligands Hoechst 33258, DAPI, and Pentamidine	NR	1	(110)
	NR	Pyrimidine-based small molecules	NR	1	(46)
	NR	Cell permeable DNA-binding agents	8	1	(47)
	ZINC database	Search used a pharmacophore model and diversity-based selection	11	2	(111)
	NCI and eMolecules	Chemical similarity search of RNA-binding ligands Hoechst 33258 and Pentamidine	75	1	(81)
	NR	Chemical similarity search of RNA-binding small molecule 1a	132	2	(87)
	LOPAC from Sigma and Diversity Set II from the NIH	–	2643	1	(49)
	1. MicroSource 2. Unknown Source	1. Natural products 2. Building blocks and scaffolds	4279	1	(42)
	NR	Diverse, drug-like primary/secondary alcohols and primary amines	20000	1	(112)
	Lead Quest	–	80000	1	(55)
	Available Chemicals Directory	–	181000	1	(54)
Corporate	Ribogene In-House Library	–	56000	1	(113)
	Merck In-House Library	Synthetic small molecules filtered for antibacterial activity	57000	1	(15)
	Parke-Davis Pharmaceutical Library	–	150000	1	(28)
FDA-Approved Drugs	–	–	1120	2	(114)
NIH Small Molecule Repository	–	–	279433 & 279988	2	(88,82)
Synthetic	Diphenylfuran Core	Core with anti-PCP activity and A-U RNA and viral RNA RRE binding	4	2	(79)
	3,5-Diaminopiperidine Core	Designed to mimic RNA-privileged scaffold 2-deoxystreptamine	8	1	(115)
	Amino Acids and Modified Nucleobases	Amino acids strengthen RNA interactions and nucleobase recognizes A-U basepairs	14	2	(116)
	Guanine Derivatives	–	16	1	(76)
	Aminoquinolone Core	Potential antibacterial, anti-HIV, and anti-HSV activity	19	4	(30)
	Curcumin, Crytolepine, Berberine, Rutaecarpine, Quinazoline, and 1-Methylquinolinium Derivatives and Natural Products	–	52	1	(44)
Theoretical	*p*-Terphenyl Small Molecules	Designed to mimic alpha-helical peptides	NR	1	(48)

NR: not reported.

4B]. The activity-based screens were performed with larger libraries (n = 8000 and 56 000 small molecules), which probed the reverse transcriptase-dependent elongation of *NRAS* mRNA by qRT-PCR (50) and the frameshifting of HIV-1 by a luciferase reporter (58). RNA binding assays included: (i) titrations against fluorescently labeled RNA; (ii) biophysical techniques (SPR and NMR); (iii) microarray immobilization; (iv) in-line probing; (v) competition dialysis and (vi) indicator displacement. We note that a lack of correlation in small molecule activity between *in vitro* RNA binding and RNA:protein displacement assays has sometimes been reported, (16,51,52) highlighting the importance of multiple assays and/or choosing the most relevant assay for a particular system. We also note that, as in all screens,

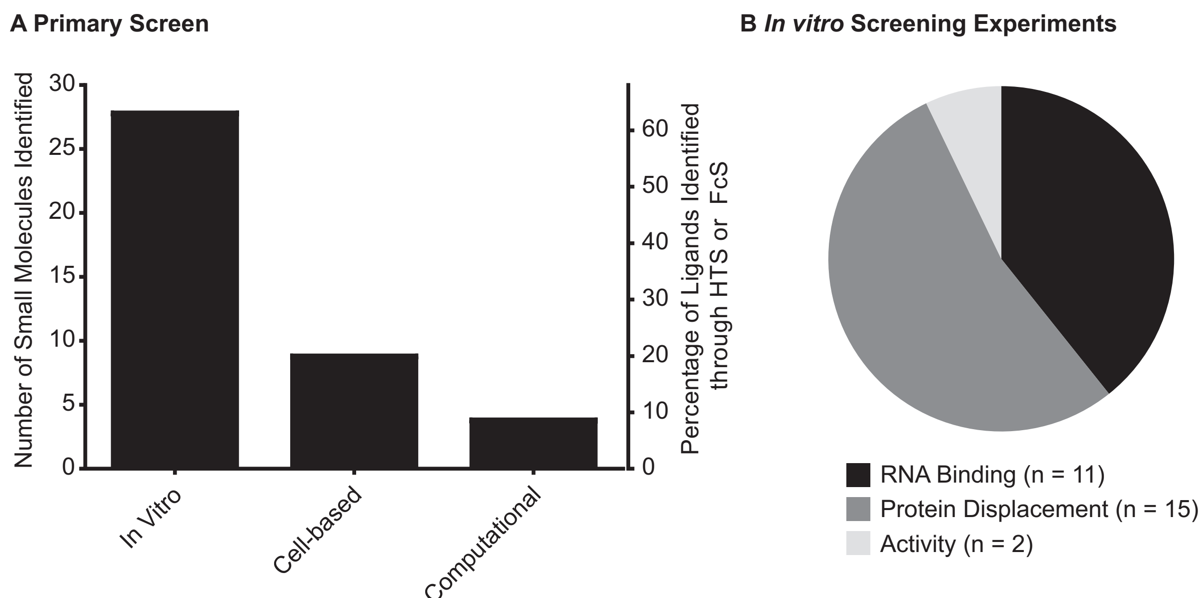


Figure 4. Primary screen tactics for identifying bioactive RNA-targeted ligands. (**A**) Of the 41 RNA:small molecule interactions discovered through primary screens using either HTS or FcS approaches, the majority originated from *in vitro* experiments ($n = 28$). The remainder were identified through cell-based assays ($n = 9$) and computational analyses ($n = 4$). (**B**) The *in vitro* primary screens were further subdivided into RNA binding, protein displacement, and activity-based experiments.

a lack of correlation can be observed between *in vitro* activity and cell culture activity. These differences can be attributed to many factors, including small molecule uptake, localization, and metabolism, specificity or off-target effects, and target availability due to binding of other macromolecules or metabolites. Nonetheless, we emphasize the success of the *in vitro* assays mentioned here in developing RNA bioactives and the valuable insights gained from other ligands discovered by *in vitro* RNA assays without reported biological activity.

Cell-based primary screening assays

Cell-based screens were the second most common primary screening assay for HTS or FcS approaches [Figure 4A] and often the preferred screen for bacterial and viral RNA targets. The exception was a splicing assay of human serotonin receptor 2C (*HTR2c*) mRNA where a green fluorescent protein (GFP) reporter was used to evaluate the inclusion or exclusion of a particular exon (53). Bacterial and viral RNA cell-based studies also utilized reporter systems such as GFP or LacZ gene as well as more traditional phenotypic screens, such as growth inhibition or cell death. In one particular example, ∼57 000 ligands were screened in a growth inhibition assay against *Escherichia coli* with and without supplementation of riboflavin (15). This differential supplementation allowed researchers to specifically probe the riboflavin pathway and confirm the FMN riboswitch was targeted by Ribocil-B. In addition, one report measured enzyme activity and antigen production in addition to cell death measurements to assess antiviral activity against HIV-1 (30). Given these successes, cell-based assays likely offer unforeseen promises as primary screening assays to discover RNA-targeted probes.

Computational primary screening assays

High-throughput and focused computational screens were used to identify four small molecules [Figure 4A]. Two small molecules were identified by docking against experimentally determined structures of HIV-1 TAR (54) and RRE RNA (48). In another example, small molecules were modeled into an X-ray diffraction structure of the *xpt-pbuE* guanine riboswitch aptamer after the native ligand was removed (46). Criteria such as geometrical constraints, hydrogen bonding patterns, and molecule planarity were used to assess the 'fit' of the ligand, leading to the selection of two small molecules, one of which had antimicrobial activity against 9 of the 15 Gram-positive bacteria species tested and was selective for species with the *guaA* gene under riboswitch control. The fourth example utilized a computationally-predicted 3D structure of the severe acute respiratory syndrome coronavirus (SARS-CoV) pseudoknot (55). A library of 80 000 small molecules was docked against the predicted structure, and the 58 highest scoring molecules were tested in an *in vitro* activity-based assay. The screen resulted in a biologically active ligand with an IC_{50} value of 0.45 μM in cell-based models. Additional advances in computational structural prediction and RNA:ligand docking will undoubtedly lead to improved computational primary screens and thus more efficient experimental screens (56,57).

Other methods of small molecule discovery

In addition to the primary screens, a database of known RNA motif:small molecule interactions, Inforna (39), was utilized to identify seven small molecules. The database was generated using a library versus library approach named 2-Dimensional Combinatorial Screening (2DCS). In this

method, small molecules are immobilized onto a microarray slide and then incubated with libraries of labeled, randomized RNA secondary structures. The bound RNAs are excised, sequenced, and assigned a fitness score using Structure–Activity Relationships Through Sequencing (StARTS). Fitness scores reflect the affinity and selectivity of a given RNA motif:small molecule interaction and are represented on a numerical scale, where a higher score represents greater selectivity. To utilize the Inforna database, a computational or experimental secondary structure of RNA is input, the 2DCS data is searched and lead molecules are proposed. This strategy identified bioactive ligands for five targets: (i) *MAPT* pre-mRNA, 1-nucleotide bulge; (58) (ii) Pre-miRNA-96, 1 × 1 internal loop (59); (iii) Pre-miRNA-18a, 1-nucleotide bulge (60); (iv) Pre-miRNA-210, 1 × 1 internal loop (16) and (v) Pre-miRNA-544, 1 × 1 internal loop (61). Other examples of ligands not identified from a primary screening assay included the selection and characterization of four metabolite analogs for riboswitch inhibition (62–65).

DISCOVERY AND DESIGN OF RNA-TARGETED MULTIVALENT CHEMICAL PROBES

In contrast to small molecules, most multivalent ligands were developed through rational design based on the secondary structure of the RNA target [Figure 5A]. Generally, development began by the identification of monovalent ligands that bound to a particular secondary structure motif(s) by screening, literature search, or using Inforna [See section '*Other Methods of Small Molecule Discovery*']. Monomers were covalently linked by selecting and optimizing appropriate sized spacers. For several multivalent ligands, the design was inspired by a crystal structure of r(CUG) sequences, leading to ligands targeting r(CUG)^{exp} in *Drosophila melanogaster* models (66). This approach linked acridine and a triaminotriazine unit, the latter of which was proposed to recognize the non-optimal base pairing of U-U mismatches by Janus-wedge hydrogen bonding. Stacking of the two units was expected to decrease nonspecific intercalative binding. This early design was optimized to yield bisamidinium conjugates that mitigated the glossy and rough eye phenotype observed in a DM1 transgenic *Drosophila melanogaster* model (67). A different approach utilized Hoechst 33258, which had been previously reported to bind a 5′CUG/3′GUC internal loop (68). Hoechst 33258 was modified to contain an azide handle and then covalently linked to a peptoid backbone via click chemistry (21). After extensive linker optimization, multivalent ligands were identified that improved r(CUG)^{exp}-related splicing defects in a mouse model of DM1.

One notable exception to the aforementioned design strategies is the use of dynamic combinatorial chemistry (DCC) [Figure 5B] (69). Several multivalent ligands were derived from a library of resin-bound, cysteine-containing monomers, which were allowed to incubate with the RNA of interest, probing thousands of multivalent ligand combinations by forming covalent yet reversible disulfide linkages. The binders with highest affinity were thus enriched and then isolated, characterized, and validated for RNA-binding. After replacing the disulfide linkage with more stable bioisosteres, the method yielded bioactive ligands for two RNAs of known structure: DM1 r(CUG)^{exp}, where statistically significant improvements in splicing were observed in mouse models (70), and HIV-1 frameshift-stimulating RNA, (71,72) where in one example the decrease in viral infectivity (EC_{50} values of 3.9 and 26 μM) correlated to frame-shifting activity (>50% at 50 μM) in cell culture (73). A powerful advantage of DCC is that multivalent ligands can be constructed without knowledge of the RNA structure, including larger and complex tertiary folds. In general, both rational design and DCC yield multivalent probes with significantly increased affinity and specificity for RNA targets relative to small molecules. While achieving high potency in biological systems with larger molecules may require more development than with traditional small molecules, the examples identified support the possibility of success.

CHEMICAL PROBE CHARACTERIZATION

Evaluating target engagement, off-target effects, potency/appropriate concentration, and other criteria is critical to understanding the quality of a chemical probe and thus any experimental conclusions (11,12,74,75). When curating the collection of chemical probes, strict benchmarks related to these criteria could not be included due to the lack of consistency within the field. In the next paragraphs, the characterization techniques utilized for RNA-targeted chemical probes will be described for each biological system and notable examples highlighted.

Bacterial and fungal systems

One of the most common validation experiments in bacterial and fungal systems was serial passage (15,31,46,62,65,76,77). In this technique, ligand resistant mutants were grown in the presence of compound and mutations were mapped by whole-genome sequencing. In addition to confirming target engagement, the results revealed off-target effects and unexpected modes of action. Further, select examples performed the serial passage experiments in multiple bacterial strains and measured binding affinity to the mutants *in vitro*, which provided added confidence in target engagement (15,31,77). In select cases where serial passage experiments did not yield mutated isolates, the ligands were tested against mutants with established variations in structure or activity (76,65). Another powerful strategy for assessing target engagement in riboswitches was phenotype rescue by addition of the native ligand (15,46). Lastly, several of the targeted RNA elements regulated the expression or translation of specific genes, which was assessed by measuring the quantity of the RNA or protein, respectively. One study went beyond measuring the expected transcripts and performed a transcriptomic microarray analysis of genes involved in many different cellular processes (46). The observed repression was consistent with riboswitch inhibition, although addition of the native ligand failed to rescue the expression of several genes, indicating a potential cellular stress response. This strategy and other genome-wide analyses can provide compelling evidence of target engagement, though it must

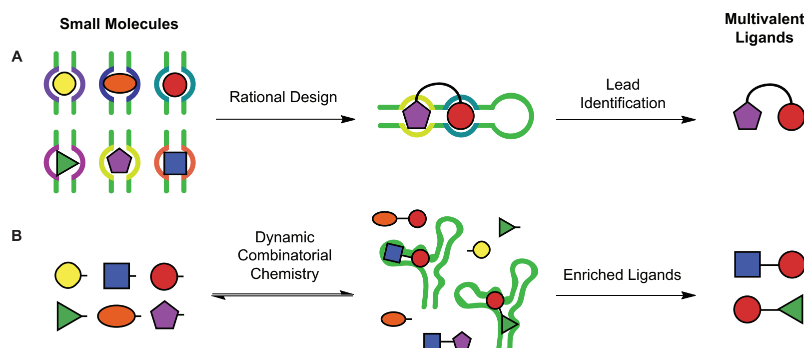


Figure 5. Design of multivalent ligands for RNA. (**A**) Using knowledge of binding interactions between small molecules and RNA secondary structures, a series of tethered ligands can be designed to probe multiple RNA structures. Lead molecules can undergo linker optimization to improve pharmacokinetic or activity properties for modulating cellular RNA functions. (**B**) In dynamic combinatorial chemistry (DCC), monomeric building blocks can form covalent and reversible linkages in solution while incubated with an RNA target of interest. Multivalent binders with the greatest affinity can become enriched in solution, identified, and then optimized for biological activity using linker replacement or chemical modifications.

be noted that target specificity does not always lead to biological specificity (12).

Viral systems

Compared to the other systems, chemical probes targeting viral RNAs were less characterized in terms of target engagement and specific activity. A select few studies validated probe activity by testing mutant versions of the virus (49,78) or closely related native viruses (30,73). Notably, one report validated probe activity with a second research group to ensure reproducibility of the biological effect (71). RNA-specific reporter systems were occasionally used to confirm on-target effects, including fusion-induced gene stimulation (79), heterologous tethering (54), and a viral protein reporter (48). A noteworthy example of assessing off-target effects was the use of RNA-Seq at increasing ligand concentrations in the absence of the target RNA (72). The experiment in HEK293T cells revealed that 53 of the 17 822 transcripts assayed had statistically significant alterations at two concentrations, potentially reflecting a cellular stress response. It is also intriguing that the most biologically potent ligand in this study was the least selective analog in an *in vitro* tRNA competition assay. This observation underlies potential differences in cellular activity versus *in vitro* binding selectivity and thus the importance of progressing multiple ligands to biological assays.

Human systems

There are several noteworthy examples of probing ligand promiscuity and/or selectivity in human systems. Multiple studies utilized genome-wide analyses to globally assess changes in miRNA, (16,17,59,60,80) mRNA, (18,50) or splicing (18,21,47,81–85) levels compared to the target RNA or process. Toward a more direct assessment, chemical cross-linking and isolation by pull down (Chem-CLIP) was utilized to investigate proximity-based engagement of RNA (86). In this method, the ligand is appended with a nucleic-acid reactive module (e.g. chlorambucil) and a biotin purification tag. After incubating cells with the modified ligand, the cells are lysed, the ligand is captured by streptavidin beads, and the bound targets are characterized by qRT-PCR or RNA-seq. This strategy as well as competitive Chem-CLIP (86) were used to characterize on- and off-target engagement of ligands that modulate repeat expansions (45,84,85,87) and miRNAs (16,17,60). In another study, dual luciferase reporter assays were utilized to compare ligand binding to four G-quadruplex structures, including the RNA of interest and three other regulatory RNAs (44). This study was one of few examples in which selectivity within a target family was assessed in cell culture.

Various controls were also used to evaluate on-target effects. For example, the impacts of chemical probes have been analyzed following siRNA knockdown of *FOXO1* mRNA (59) and following inhibition of the mTOR signaling pathway modulated by miRNA-544 (61). Another example overexpressed miRNA-210 and assessed the effect of the chemical probe on the phenotype (16). Likewise, a ligand targeting $r(CUG)^{exp}$ was tested with the RNA under conditional expression (88). Another notable example used RNA immunoprecipitation (RIP) to detect RNA-binding at increasing concentrations of ligand and identified a dose-dependent response (19). For precipitation, a G-quadruplex specific antibody, BG4, was utilized and the complex was characterized by two complementary methods: dot blotting and qRT-PCR. Another important control, particularly for human systems, was to replicate on-target effects in at least two cell lines, though this control was performed for only a limited number of chemical probes (44,53,47,81,84).

CONCLUSIONS AND PERSPECTIVES

For many years, RNA has been labeled as 'undruggable' or 'impossible to probe selectively'; however, the reports described herein demonstrate the substantial progress that has been achieved in the past four years. This includes the development of chemical probes for 33 unique RNA elements, though the number of 'targetable' RNA elements is certain to vastly exceed this list (5,6,23,89). Those in Archaea, for example, are unrepresented in reports of RNA-targeted chemical probes, despite the established importance of small regulatory RNAs in archaea metabolism, morphology, and adaption to extreme conditions (90,91).

Furthermore, there are several RNAs implicated in diseases for which novel treatment strategies are needed, including insect-borne viruses (23), genetic disorders (92), and metastatic cancers (5,6) as well as bacterial targets amid the antibiotic-resistance crisis (89,93). There are also many opportunities for RNA-targeting in fungal systems (94), especially as fungal infections are experiencing a rise in cases and a therapeutic plateau (95). Fundamentally, the development of chemical probes will allow for the rapid and reversible interrogation of novel and complex RNA biology in ways not attainable by knockdown and genetic approaches (74).

While the potential benefits of selective RNA targeting are staggering, approaches toward chemical probe development must thoughtfully consider a number of variables, including transcript abundance and tissue-specific expression of the RNA target. By total mass and number of molecules, rRNA and tRNA account for greater than 90% of total cellular RNA in humans (96), and thus mRNAs and other ncRNAs exist in a much lower abundance (1,96,97). Even within these low abundance transcripts, copy numbers can vary widely within and across cell types with reports of mRNA levels spanning four orders of magnitude and some ncRNAs averaging less than one copy per cell (1,97,98). A direct impact of RNA expression levels was recently reported, in which the authors proposed that ligand occupancy of the miRNA target was driven by the relative abundance of structurally similar RNA elements (16). RNA with well-defined function and that are highly expressed thus represent low-hanging fruit within the field (6,99).

The selection of a library and primary screening strategy is also critical for the success of chemical probe discovery. For RNA targets with known structure and/or ligands, the use of focused screening libraries (35,36) has proven to be an efficient strategy, though this approach generally limits the chemical diversity of the library. Advances such as the identification of more RNA-privileged scaffolds (39) and biologically relevant RNA chemical space (22,100) will facilitate the discovery of chemical probes for additional RNA targets. These efforts could be expedited by additional fragment- or natural product-based screening to access vast chemical space with fewer ligands (41) and to probe known biologically relevant chemical space, respectively (101). Additional high-throughput screens could likewise discover novel chemotypes, though the expense may exceed the resources available in academia, where focused approaches are more attainable. Continued progress in the development and refinement of computational tools will also aid in expanding the boundaries of focused screening and structure-based design;(56,57) although the latter will also depend upon advancements for accurately determining atomic resolution structures (8,9,37,38). In addition, *in vitro* or cellular activity-based assays that probe well-studied RNA functions (e.g. splicing, translation, or processing) may be more practical starting points to identify chemical probes. This includes screening strategies described herein as well as others recently developed (102,103). Finally, numerous opportunities exist to build upon the established multivalent targeting strategies discussed above, particularly the application of dynamic combinatorial chemistry to RNAs of unknown structure.

When using a chemical probe in a biological system, the quality and specificity of the probe must be well characterized to draw accurate and meaningful conclusions, as recently highlighted by several preeminent chemical biologists (11,12,74,75). Evaluation of the chemical probes revealed a diverse spectrum of characterization techniques with few ligands meeting the traditional criteria for robust chemical probes, and not all of these gaps can be attributed to a lack of relevant tools. For example, characterization inconsistencies include incomplete reports of cytotoxicity and a lack of attention to cell permeability and localization. In addition, many *in vitro* assays are accessible to help establish the potency and selectivity of a probe in multiple experiments, and these assays should include evaluation against both specifically mutated targets and a number of other structured RNAs. Any cell-based observations should be reproducible in multiple cell lines and validated in the absence of the target by utilizing siRNA or CRISPR-Cas technologies. Further, on-target effects should be established by using a number of spatial-based experiments (12). These include the biochemical methods described herein (e.g. Chem-CLIP or RIP) and novel applications of other technologies such as in-cell chemical probing (104,105) to observe changes in RNA secondary structure upon ligand binding or photoaffinity labeling (106) to assess target engagement under temporal control. If plausible, serial passage experiments followed by deep sequencing should also be performed, even in human systems (107), to identify ligand-escaping mutations to confirm target engagement and/or off-target effects. Lastly, it is critical to use an inactive analog and an active analog from a different chemical class, if feasible, to draw conclusions regarding the targeted biology. Moving toward these standards will be crucial for the RNA-targeting field to avoid the scientific pollution that has plagued many others (11,12,74).

For comparison, we note that over a decade ago chemical probe discovery for another 'undruggable' target, protein:protein interactions, was in its infancy with only 19 known small molecule inhibitors (108). However, novel advances in screening approaches and design strategies led to the rapid discovery of thousands of protein:protein interaction inhibitors with several entering the clinic and most also breaking the rules of 'drug-like chemical space' (109). In the next five years, we anticipate that the innovations described herein and the ones yet to be discovered will lead to a similar surge in reports of RNA-based chemical probes and therapeutics. We also expect that novel and well-characterized chemical probes will allow RNA biologists to uncover many more exciting and unanticipated roles for RNA, propelling us into the next phase of the RNA revolution.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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