Design of RNA-binding proteins and ligands

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The rapidly expanding database of RNA structures and protein complexes is beginning to lead to the successful design of specific RNA-binding molecules. Recent combinatorial and structure-based approaches have utilized known nucleic-acid-binding scaffolds from both proteins and small molecules to display a relatively small set of functional groups often used in protein–RNA recognition. Several studies have shown that the tethering of multiple binding modules can enhance RNA-binding affinity and specificity, a strategy also commonly used in DNA recognition.

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Abbreviations

ARM	arginine-rich motif
BIV	bovine immunodeficiency virus
CD	circular dichroism
RRE	Rev response element
RRM	RNA recognition motif
TAR	transactivation response element

Introduction

The ability of RNA molecules to fold into complex three-dimensional shapes and the importance of RNAs to many biological processes have generated great interest in designing sequence-specific RNA-binding molecules. The design process can be useful for testing our understanding of the basic principles of nucleic acid recognition, as well as for developing new types of drugs. The design of sequence-specific DNA binders has been pursued for quite some time, with exciting recent successes utilizing zinc finger proteins to target sites in the major groove [1–3] and polyamides to target sites in the minor groove [4,5]. The design of RNA binders has lagged behind, largely due to a paucity of structural information on RNAs and RNA–protein complexes. Fortunately, the past few years have produced an explosion of crystallographic and NMR structures, and this, together with the information gained on designing DNA binders and the development of new combinatorial tools, has resulted in a few cases of successfully designed peptide or small-molecule RNA binders.

Conceptually, RNA targets have characteristics in common with DNA, as well as with protein. Most obviously, both RNA and DNA are nucleic acids, composed of bases that provide abundant hydrogen-bonding moieties and that are tethered via the charged sugar–phosphate backbone. Unlike DNA, however, RNAs fold with a much larger repertoire of possible base pairings and tertiary structural motifs, sometimes displaying pockets, deep grooves and intricate surfaces that resemble protein structures more than DNA. In principle, this diversity of shape provides opportunities to design ligands that bind with high affinities and specificities using structure-based strategies. However, one major obstacle is that RNAs often rearrange or fold only upon protein binding, and thus the target site, or at least one possible conformation, may not be well defined in the absence of ligand.

This review highlights recent progress towards designing specific RNA-binding proteins and ligands using 'rational' structure-based and 'semirational' combinatorial approaches. We emphasize the types of scaffolds and functional groups used, and discuss attempts to enhance affinity and specificity by tethering together multiple binding modules.

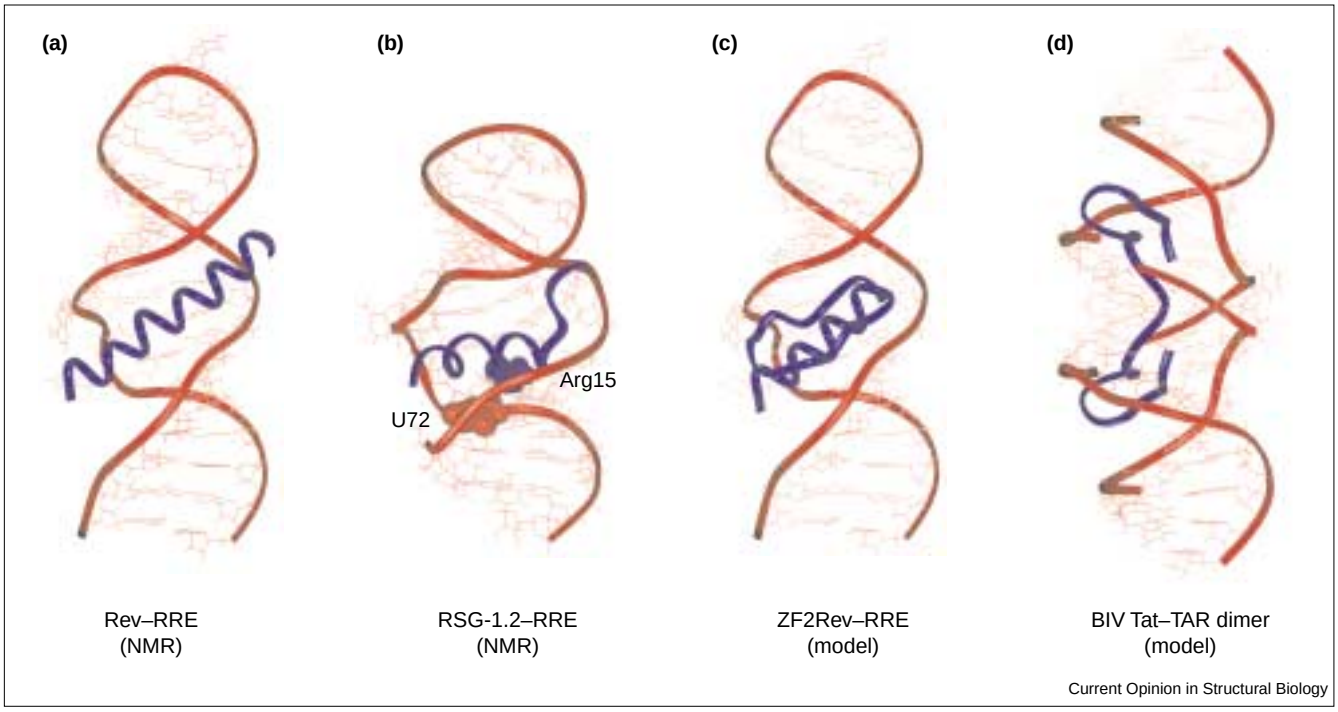
Protein and peptide scaffolds

One of the first protein design efforts focused on the RRM (RNA recognition motif, or RNP domain), the most commonly found RNA-binding domain. This approximately 100 amino acid domain adopts a mixed α/β architecture and typically binds RNA via its β-sheet surface. A combinatorial phage display library containing nine partially randomized amino acids in one RRM domain from the U1A protein was screened for high-affinity binders to its U1 snRNA hairpin binding site [6]. In addition to deriving an amino acid consensus sequence for U1 RNA binding, one variant (a valine to alanine substitution) was found to bind with twofold higher affinity than the wild-type RRM, despite the small size of the library screened (512 sequences). Based on a U1A–RNA co-crystal structure, the altered valine does not directly contact the RNA and thus the change may alter the binding orientation of the domain. The ubiquitous nature of the RRM, the apparent ease of finding tight-binding variants and the wide diversity of RNA sites recognized (single-stranded and structured RNAs) suggest that the domain may provide a good framework for engineering desired specificities. Other common, modular RNA-binding domains, such as KH and zinc finger domains, may provide good additional starting scaffolds.

Zinc fingers

Zinc finger domains are modules of approximately 30 amino acids containing an α helix and a β sheet coordinated by a zinc ion, and often are arrayed as tandem copies in proteins. Most zinc finger proteins bind to specific DNA sequences, recognizing approximately three base pairs per finger via the insertion of an α helix into the major groove, but some fingers also bind RNA. A large number of combinatorial library experiments have demonstrated that zinc fingers are excellent modules for designing sequence-specific DNA binders and some approximate rules for binding have been elucidated, whereby particular positions of the

Figure 1



Designed arginine-rich RNA-binding peptides. (**a**) The wild-type Rev peptide binds as an α helix to the RRE [14], (**b**) the selected RSG-1.2 peptide binds in a helix-turn-extended chain conformation [22••,23•] and (**c**) an RRE-binding zinc finger engineered with the Rev helix is presumed to bind similarly to Rev [13••]. (**d**) A structure-based approach was used to design a dimeric BIV Tat–TAR complex [29••]. Peptides are shown in blue and the RNA in orange.

α helix bind to specific bases, although no strict recognition code is observed [1–3,7]. Zinc finger libraries now are being exploited to identify specific RNA binders.

In one phage display experiment, zinc fingers were identified that bind to a single G•A base pair in an unusual helical context. In this case, one zinc finger randomized at several positions of the α helix was placed between two DNA-binding zinc fingers and RNA triplets were engineered between two flanking DNA helices used to anchor the two flanking fingers [8]. Specific G•A binders were isolated that were also found to bind to a C•A base pair, presumably because the G•A and C•A pairs disrupt the RNA portion of the helix in similar ways. It is well appreciated that the major groove of an A-form Watson–Crick RNA helix is relatively inaccessible to proteins and requires bulges or noncanonical base pairs to allow binding [9]. Consistent with the need for noncanonical base pairs, selection experiments performed using DNA helices engineered with only Watson–Crick RNA triplets yielded no specific binders.

Friesen and Darby [10] used combinatorial zinc finger libraries first to characterize the amino acid requirements for the binding of TFIIIA finger 4 to 5S rRNA, in the context of the four zinc fingers known to bind this RNA [10]. The pattern of required residues in the α helix was at least partially related to that observed for DNA-binding fingers, suggesting that RNA-binding fingers also may use a 'recognition helix' to dock in the RNA major groove. They next identified two-finger proteins that recognize either 5S rRNA or the HIV-1 Rev response element (RRE) IIB hairpin from a library in which the linker and nine helical positions in each finger (both based on TFIIIA finger 4) were randomized [11]. After DNA shuffling to enhance the library complexity, binders to both RNAs were found with affinities comparable to their wild-type protein partners. For the RRE IIB binders, the C-terminal finger was typically enriched in arginine and lysine residues, whereas the N-terminal finger was enriched in acidic or polar residues. The C-terminal finger on its own recently has been shown to bind RRE IIB with a K_d value of approximately 20 nM and, assuming that the fingers bind independently, contributes approximately 95% of the total binding energy [12••]. The binding of this dominant finger requires the same nucleotides in RRE IIB as Rev peptide binding, which uses an arginine-rich α helix to recognize a distorted major groove (see below and Figure 1). Thus, it seems reasonable that the selected zinc finger helix may be positioned similarly. Further phage display experiments using two-finger libraries and a larger, 234-nucleotide RRE element instead of the isolated IIB hairpin (the highest affinity Rev-binding site) resulted in subnanomolar binders that presumably recognize additional regions of the RNA [12••].

In addition to the combinatorial library experiments, a structure-based approach has been used to design an

RRE-IIB-binding zinc finger (Figure 1). The arginine-rich α helix of Rev, which must be structured before RNA binding, was modeled into a zinc finger domain from the Zif268 protein, conserving the six amino acids critical for RRE binding and the two histidines required for zinc coordination [13••]. This zinc finger–Rev hybrid, in which the helix has been locked into its folded state, bound RRE IIB in a zinc-dependent manner with an affinity and specificity similar to that of a Rev peptide stabilized in its helical conformation by blocking its termini. Mutation of cysteine residues required for zinc coordination and proper folding of the finger disrupted binding, and mutation of amino acids or nucleotides critical for the Rev–RRE interaction also disrupted binding, suggesting that the designed zinc finger–Rev peptide hybrid binds the RRE in a manner similar to Rev. The designed RRE-binding finger apparently binds differently compared with DNA-binding zinc fingers. The sidechains in the helix that presumably contact the RNA (assuming the same binding orientation as in the Rev–RRE IIB NMR structure) display a very different pattern to the –1, +2, +3 and +6 positions that usually make base-specific contacts in the helices of DNA-binding fingers [1–3]. In addition, the helix is expected to be more extensively surrounded by RNA when bound in the deep and distorted RRE major groove [14,15].

Arginine-rich motif

In contrast to the prefolded RRM or zinc finger domains described above, efforts to design novel RNA-binding peptides have focused on the arginine-rich motif (ARM), a structurally diverse peptide framework that requires RNA binding to adopt a stable structure [16,17]. The ARM is typically less than 20 amino acids in length and has been shown to bind RNA in α-helical, β-hairpin and extended conformations, depending on the peptide sequence and RNA site. In some cases, one peptide can adopt different conformations when bound to different RNA sites [18,19]. Arginine-rich peptides have provided good scaffolds for design and selection experiments because they are short, conformationally diverse and can bind RNAs with high affinities (picomolar) and specificities (displaying >1000-fold preferences for cognate binding sites). Although the dominant contributions of the arginine sidechains may differ among complexes, the potential of the guanidinium group to make electrostatic, hydrogen bonding, cation–π and π-stacking interactions makes arginine a versatile moiety for RNA recognition, also leading to its extensive use in small-molecule design, as described below.

In an early effort to identify tight RRE binders, arginine-rich peptide libraries were screened using a bacterial anti-termination assay that monitors RNA–protein interaction [20]. A library in which nine positions were randomized with non-helix-promoting amino acids (arginine, serine and glycine) yielded peptides that bound RRE IIB with equal or higher affinities and specificities than the wild-type Rev peptide. Two additional rounds of mutagenesis resulted in a peptide, RSG-1.2 (DRRRRGSRPSGAERRRRRAAAA), that bound RRE IIB with 7-fold higher affinity and 15-fold higher specificity than the Rev peptide and efficiently displaced the full-length Rev protein from the RRE *in vitro* and inhibited Rev function in mammalian cell reporter assays [21]. CD experiments indicated that the RSG-1.2 peptide was partially helical and unexpectedly the helix was stabilized by introducing proline in the middle of the peptide, leading to the suggestion that its C-terminal region might be helical and preceded by a turn. Recent solution structures of RSG-1.2 bound to RRE IIB [22••,23•] confirm the unique, partially nonhelical fold and show a very different binding mode to Rev (Figure 1). The RRE IIB structure is similar to that seen in complex with Rev, with the formation of G•G and G•A base pairs, but the nature of the amino acid–RNA contacts is quite different. A dominant interaction in the Rev–RRE complex involves hydrogen bonding between the G•A pair and a critical asparagine [14,15], which is lacking in RSG-1.2. The detailed contacts deduced by Patel and co-workers [22••] suggest that some of the additional affinity and specificity of RSG-1.2 may result from hydrophobic contacts to Ala12 and from stacking or cation–π interactions between Arg15 and the unpaired U72 of an internal bulge (Figure 1). U72 is disordered in the Rev peptide–RRE complex, but becomes fixed in the RSG-1.2 complex [22••].

Other combinatorial experiments have yielded peptides with specificity for particular non-Watson–Crick base pairs. In another RRE-binding screen performed in mammalian cells, in which four positions were randomized within a stretch of 14 arginines, a single glutamine embedded in the polyarginine context was found to bind RRE IIB with higher affinity than Rev [24]. It is presumed that the glutamine makes a contact analogous to the Asn–G•A hydrogen-bonding interaction of Rev. In another case, a phage display library with 10 randomized amino acids flanked by RGG motifs was used to identify binders to a $tRNA^{Ala}$ microhelix containing a G•U wobble pair, based on the recognition properties of the alanyl-tRNA synthetase [25]. Peptides were found that distinguish the G•U wobble pair from I•U, G•C and U•G in the minor groove, and required a serine and glutamic acid. It is suggested that the acidic glutamic acid sidechain may be used to sense the partial positive charge of the exocyclic 2-amino group of guanine in the minor groove. Establishing exactly how the G•A and G•U pairs are recognized in these two cases will require additional structural studies.

Tethered recognition modules

A very successful approach used to enhance the affinity and specificity of DNA-binding proteins has been to link multiple binding domains together, as exemplified by many zinc finger studies [26–28]. In principle, binding affinity can be increased substantially simply by enhancing the local concentration of extra modules once the first has bound [27]. One successful structure-based approach has utilized the NMR structure of the bovine immunodeficiency virus (BIV) Tat–transactivation response element (TAR) complex

to design a dimeric interaction involving two β-hairpin peptides bound to two adjacent RNA sites [29••] (Figure 1). The dimeric complex is formed with 10-fold higher affinity than the monomeric complex. The enhancement is more modest than typically observed when DNA-binding modules are tethered and may reflect the need for the unstructured dimeric peptide to fold into its β-hairpin conformation upon RNA binding or accessibility problems caused when extra Watson–Crick base pairs, known to decrease major groove accessibility [9], are introduced in the dimeric arrangement [29••]. These problems emphasize two key differences between the design of RNA and DNA binders: conformational changes and induced-fit interactions (in the protein or nucleic acid) tend to be more extreme upon RNA binding, and considerations of RNA secondary and tertiary structure are paramount. Nonetheless, the initial tethering results for the dimeric BIV peptide and zinc finger complexes (described above) help establish the soundness of the principle for RNA. Further optimization of the individual units and linkers, and mixing and matching of the various peptide, zinc finger, RRM or other modules may be expected to generate tighter and more specific tethered complexes. Related approaches are also being applied to ligand design, as described below.

Small-molecule scaffolds

There has been substantial progress in designing RNA-specific ligands, in part due to the explosion of structural information on RNAs and RNA complexes. The general area of RNA–ligand interaction has been the subject of several recent reviews [30–34]. Here, we highlight just a few examples of small-molecule binders that reinforce some of the design and recognition concepts described for proteins and peptides.

Aminoglycosides

Naturally occurring aminoglycoside antibiotics have been known for many years to target ribosomal RNAs and, more recently, to target other structured RNAs. Detailed structures are now available for several complexes, including with ribosomal subunits (see [30–34]). The general RNA-binding properties of aminoglycosides may be attributed, at least in part, to their constellations of cationic groups around the various rings that can make favorable electrostatic contacts and hydrogen bonds to the RNA (see Figure 2, molecules v, vi and vii). As with most protein-binding sites, aminoglycoside-binding sites typically are found in RNA helical domains containing irregularities, such as bulges, non-Watson–Crick base pairs or base triples, that widen the major groove and allow access. It is interesting that neomycin appears to bind to the minor groove of HIV-1 TAR and induce a conformational change that inhibits Tat protein binding in the major groove [35,36]. The relatively flexible aminoglycoside scaffold has been used extensively to generate a large number of derivatives and libraries, and detailed structure/activity relationship (SAR) analyses have been performed in several cases (see [30–34]). We describe a few examples below in which the scaffold has been used to display protein-like sidechains or in which binding units have been tethered.

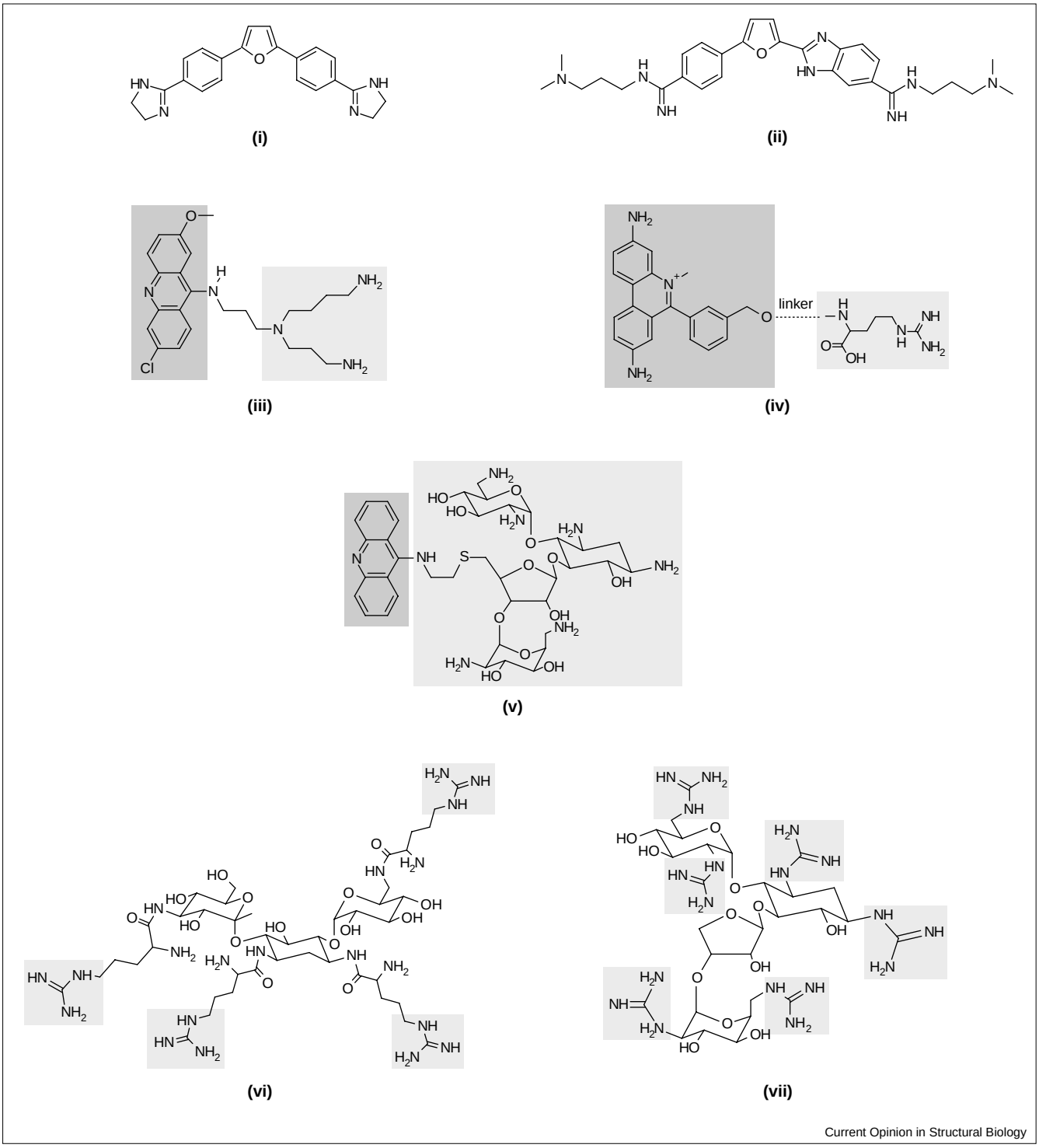
Heterocycles and other scaffolds

A large variety of compounds has been found to bind RNA specifically or nonspecifically, including heterocycles, intercalators and polycations; some of these motifs have been exploited in drug design efforts (see [30,31,33,34,37,38]). Several diphenylfuran derivatives known to interact with DNA in the minor groove have been shown to bind to HIV-1 TAR, probably in the major groove, with a bi-imidazole version (Figure 2, molecule i) showing the highest affinity [39]. A related tetracationic phenyl-furanbenzimidizole compound (Figure 2, molecule ii) binds to HIV-1 RRE in the minor groove, apparently with a 2:1 drug : RNA stoichiometry in a dimeric arrangement [40••]. NMR and footprinting experiments suggest that the RNA structure is largely unchanged upon binding the compound. Other aromatic heterocyclic derivatives also bind to the RRE, but all appear to use rather different binding modes compared with Rev or neomycin, an RRE-binding aminoglycoside [38]. Attempts also have been made to design various peptidomimetics of arginine-rich peptides. Oligourea and oligocarbamate backbone mimetics of the ARM of Tat bind to HIV-1 TAR *in vitro* and show inhibition of Tat activity in tissue culture assays at micromolar concentrations [41]. Cyclic derivatives of the Tat ARM have been found to inhibit Tat activity, as well as to disrupt the Rev–RRE interaction [42,43]. In addition, tripeptides composed of D- and L-amino acids selected from a combinatorial library were found to bind TAR RNA and inhibit Tat activity [44]. At least one of the tripeptides binds to the bulge of TAR, but induces a conformational change in the RNA that is different from that induced by Tat binding, as deduced from NMR experiments.

Tethered modules

As with RNA-binding proteins, one promising approach to enhance the affinity and specificity of RNA-binding ligands is to mix and match various binding modules. HIV-1 TAR binders were found by tethering aromatic moieties, such as acridine, to various cationic moieties, such as spermidine (Figure 2, molecule iii), thereby providing functional groups for both stacking and charge interactions [45]. NMR experiments suggest that the hybrid molecule binds to the bulge region of TAR, as does the guanidinium group of a single arginine residue in the ARM of Tat, which simultaneously hydrogen bonds to a guanine base, interacts with adjacent phosphate groups and may also stack on a base [17]. A hybrid molecule with arginine tethered to an ethidium moiety (Figure 2, molecule iv) also appears to bind to the same region of TAR, based on RNase footprinting experiments, and inhibits HIV-1 replication at micromolar concentrations [46]. Aminoglycosides also have been used in tethered arrangements. Dimeric versions of several aminoglycosides have been shown to inhibit the activity of a *Tetrahymena* ribozyme 1–3 orders of magnitude more effectively than their monomeric counterparts [47].

Figure 2



A few examples of RNA-binding ligands: (i) TAR-binding heterocycle [39]; (ii) RRE-binding heterocycle [40••]; (iii) TAR-binding acridine–polyamine [45]; (iv) TAR-binding ethidium–arginine [46]; (v) RRE-binding acridine–neomycin [48••]; (vi) TAR-binding kanamycin–tri-arginine [49•]; and (vii) RRE-binding guanidinoglycoside [50•]. The linked modules of molecules iii, iv and v are shaded, and the guanidinium moieties of molecules vi and vii are shaded.

Neomycin conjugated to acridine (Figure 2, molecule v) inhibits the Rev peptide–RRE interaction at low nanomolar concentrations and more than an order of magnitude more effectively than neomycin alone [48••]. Interestingly, the acridine moiety of this conjugate may bind near U72, the same bulged nucleotide that becomes fixed in structure

when bound to the RSG-1.2 peptide (Figure 1). Arginine sidechains have been tethered to gentamycin and kanamycin (Figure 2, molecule vi), resulting in molecules that bind HIV-1 TAR and exhibit extended footprints that include the bulge region [49•]. Guanidinium groups have been added chemically to aminoglycosides, converting them into guanidinoglycosides (Figure 2, molecule vii), which show enhanced affinity and specificity for HIV-1 RRE compared with their amino precursors [50•]. The recurrent use of the arginine guanidinium group for RNA recognition in the context of proteins, peptides and small molecules further emphasizes the versatile nature of the sidechain.

Future prospects

Combinatorial and structure-based design approaches both are expected to continue generating novel and interesting types of RNA-binding molecules. Affinity and specificity may be enhanced in some cases by tethering multiple binding units together, as nature has demonstrated repeatedly in proteins containing zinc finger, RRM and KH binding modules. The development of new protein and ligand scaffolds will be important for generating a more diverse set of building blocks for the design process. Although not covered in this review, the development of new computational tools and screening methods, and more detailed thermodynamic and kinetic characterization of RNA complexes also will be essential. Detailed structural information on novel complexes emerging from the design process is expected to shed new light on the fundamental principles of RNA structure, recognition by proteins and ligands, and the roles of induced fit.

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The authors demonstrate that the guanidinium group of arginine also can enhance affinity for RRE RNA (see [49•]).