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A Novel Solid-Phase Assembly for Identifying Potent and Selective RNA Ligands**

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Replication of the human immunodeficiency virus (HIV-1) requires an ordered pattern of viral gene expression.[1] This process is dependent upon the association of Rev, an essential viral regulatory protein, with its respective RNA binding site, the Rev-response-element (RRE).[2] The RRE is necessary for the active export of unspliced genomic viral RNA from the nucleus and serves as part of the envelope – protein coding sequence.[2] This dual function makes the Rev – RRE binding event an attractive target for therapeutic intervention because the evolution of resistant variants may be prevented or impeded. Small molecules that specifically bind the RRE and preclude or competitively displace the Rev protein are therefore promising antiviral candidates.[3–5]

Aminoglycoside antibiotics have been shown to competitively block the binding of the Rev protein to the RRE, thus providing an important precedent for the use of low molecular weight ligands to target viral RNA sites.[3, 6] These natural antibiotics, however, bind the RRE with relatively low affinity and specificity.[7] Since aminoglycosides and most other RNA ligands appear to recognize certain RNA folds, rather than specific RNA sequences, the design and discovery of RNA ligands is a challenging and empirical process.[7b] Commonly employed RNA-binding assays are limited in their ability to probe both the affinity and specificity of potential binders.[7a, 8, 9] New approaches that allow the rapid determination of both the RNA affinity *and* specificity of small molecules will assist in the discovery of new lead compounds and advance the understanding of RNA recognition. To this end, we report the assembly of an immobilized RNA – protein complex and demonstrate its application to the discovery and characterization of new RNA binders.

The high-affinity Rev binding site within the RRE is the purine-rich bulge shown in Figure 1.[10] The arginine-rich segment, $Rev_{34–50}$, binds the RRE with a dissociation constant similar to that of the full-length Rev protein.[11] We have developed an assay based on the competition between potential RNA binders and a fluorescent Rev peptide ("Rev-Fl") for binding to an immobilized RRE fragment. The assay identifies small molecules that specifically interfere with Rev – RRE binding (Figure 1). Ligands that bind to the

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Supporting information for this article is available on the WWW under http://www.wiley-vch.de/home/angewandte/ or from the author.

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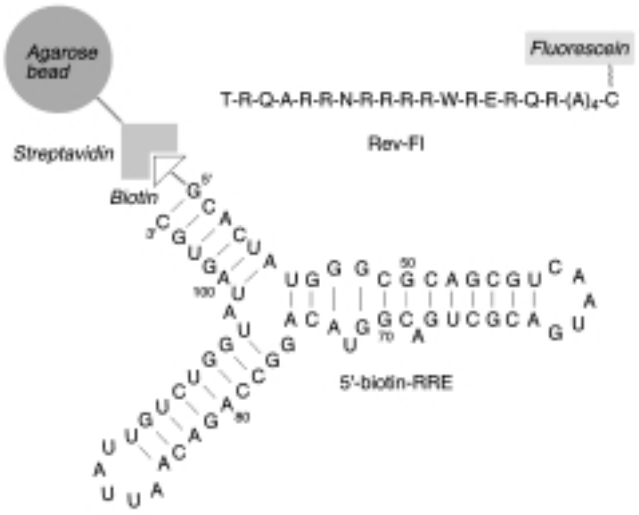


Figure 1. Schematic representation of the immobilized RNA–peptide assembly, including the proposed secondary structure of the biotinylated RRE and the fluorescently tagged arginine-rich RNA binding domain of the Rev protein ("Rev-Fl").

RRE will effectively compete with Rev-Fl, thus releasing the peptide into solution. The amount of fluorescent peptide in solution or remaining on the solid support is then quantified. The assay's stringency level can be tuned by adding nucleic acids, proteins, or any other competing species. Nonselective ligands will be scavenged by competitor molecules present in solution. Only when a highly selective RRE binder is present will a positive fluorescence signal be elicited in solution. The global selectivity of the inhibitor can then be assessed based upon the identities of the competing species.

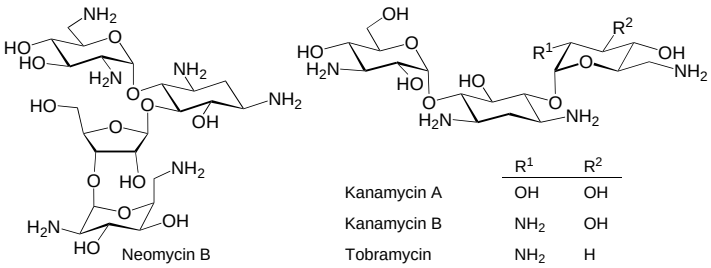
The components of the assembly are shown schematically in Figure 1 and include: a) insoluble agarose beads covalently modified with streptavidin, b) a biotinylated RRE fragment, and c) a fluorescein-labeled Rev fragment.[12, 13] Assembly of these three components forms an immobilized ternary complex, in which the biotinylated RRE binds to streptavidin, and the fluorescein-tagged Rev specifically binds to the RRE.[14] The Rev-Fl–RRE dissociation constant ($K_d = 2.5$ nM) has been found to be the same in solution as on the solid support.[15]

To demonstrate the effectiveness and dependability of the solid-phase assay, aminoglycoside antibiotics, a thoroughly investigated family of RRE binders, have been examined.[7b] Table 1 summarizes the IC_{50} values obtained by the solid-phase assay in comparison with those obtained by fluorescence anisotropy measurements.[16] An excellent correlation is observed between the two methods for the aminoglycoside family. The assay faithfully reproduces the known trend in RRE affinity of these natural products (neomycin B > tobramycin > kanamycin B > kanamycin A).[3, 7a] Repeating the experiments in the presence of nucleic acid competitors confirmed the selectivity typically exhibited by aminoglycosides.[7b] A low affinity to double-stranded DNA is evident from essentially identical IC_{50} values in the presence and absence of plasmid DNA, and promiscuous RNA binding is manifested as higher IC_{50} values in the presence of transfer RNAs.[17]

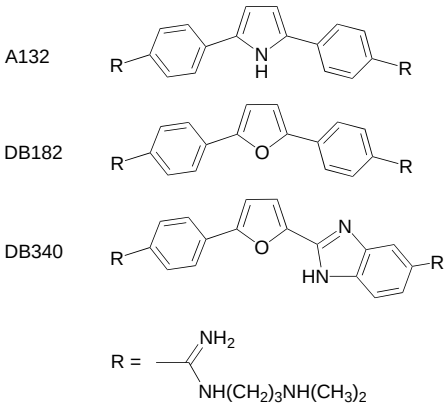
Table 1. IC_{50} values [μM] as determined by fluorescence anisotropy and the novel solid-phase assembly.[a]

Compound	Aniso-tropy	Solid phase	Solid phase + DNA	Solid phase + tRNA
neomycin B	7	7	8	20
tobramycin	47	45	50	100
kanamycin B	80	90	90	170
kanamycin A	780	750	750	1200
DB340	0.3	0.1	1.3	0.7
DB182	1.5	0.4	4	0.8
A132	10[b]	1.2	10	3
Δ-$[Ru(bpy)_2(eilatin)]^{2+}$	1.0	2.0	10	2.0
Λ-$[Ru(bpy)_2(eilatin)]^{2+}$	1.0	2.0	50	2.0
$[Ru(bpy)_3]^{2+}$	170[b]	> 10000	n.d[c]	n.d[c]

[a] See Supporting Information for experimental details and conditions. The standard deviations of all results are within ±25% of the reported value. [b] Significant fluorescence quenching occurred during the course of titration. See ref. [20]. [c] n.d. = not determined.



To examine the versatility of the assembly-based assay the competitive binding of polycyclic aromatic amidines to the RRE has also been examined.[18] This family of ligands is more potent than aminoglycosides (see Table 1). Their previously reported trend in RRE affinity is again accurately reproduced



(DB340 > DB182 > A132).[18] A tenfold increase in IC_{50} values in the presence of competing DNA indicates that these compounds also have a relatively high affinity for double-stranded DNA.[17] This result clearly illustrates how the observed potency of an inhibitor is influenced by its selectivity.

Discrepancies between IC_{50} values determined from the solid-phase assay and the values obtained by fluorescence anisotropy measurements reflect a general problem associated with applying fluorescence anisotropy to binders that are either fluorescent or fluorescence quenchers (Table 1).[19] The

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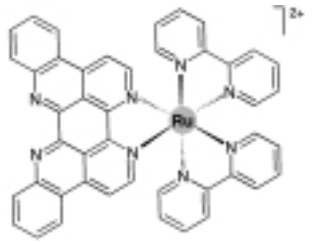
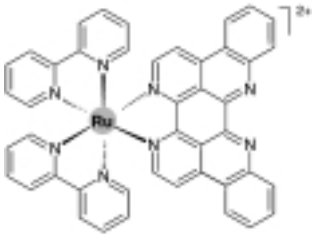
solid-phase assay easily adapts to this problem: if an inhibitor interferes with Rev-Fl emission, the supernatant (containing excess inhibitor and displaced Rev-Fl) is simply washed away, and the fluorescence remaining on the solid support is quantified.[13] Gel mobility shift assays confirm that, for these inhibitors, IC_{50} values obtained from the solid-phase assembly are more accurate than the IC_{50} values derived from fluorescence anisotropy measurements.[13, 20]

The novel solid-support assembly has also been used to identify and characterize new Rev–RRE inhibitors. The enantiomeric, octahedral metal complexes Δ- and Λ-$[Ru(bpy)_2(eilatin)]^{2+}$ are potent inhibitors of the Rev–RRE complex and exhibit intriguing selectivities.[21] The fluorescence anisotropy and the solid-phase assays indicate that both enantiomers have the same affinity for the RRE (Table 1). In the presence of DNA, however, the Λ enantiomer looses its potency by a factor of 25, while that of the Δ enantiomer is reduced only by a factor of 5. This result suggests that the Λ enantiomer has a significantly higher affinity for double-stranded DNA than the Δ enantiomer. The potency of neither is affected by the presence of tRNA. This observation illustrates that these metal complexes have the opposite nucleic acid selectivity as compared to the aminoglycoside antibiotics family.



Δ-$[Ru(bpy)_2(eilatin)]^{2+}$



Λ-$[Ru(bpy)_2(eilatin)]^{2+}$

Both selectivity and affinity are crucial for the design and evaluation of new RNA–protein inhibitors.[5, 7b] Assembly of the immobilized Rev–RRE complex has generated a simple, rapid, and quantitative assay that facilitates the discovery and characterization of new RNA binders. Since competitive binding studies can be conducted in complex mixtures of biomolecules, this assembly provides a powerful tool for the characterization of the inhibitor's affinity and selectivity. Critical evaluation of the assay has shown that the expected inhibitory activities of diverse and potentially problematic families of molecules are consistently and faithfully reproduced. This prototypical approach is amenable to automation and high-throughput screening and is applicable to other RNA–protein complexes.

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[12] Modified Rev_{34-50} peptides of the sequence $^{34}_{suc}$TRQARRNRRRRWRERQR^{50}AAAAC$_{am}$ have been employed for all experiments. The conjugation of fluorescein to the cysteine residue results in "Rev-Fl", which has approximately the same affinity to the RRE as the unlabeled Rev peptide ($K_d = 1.2$ nM).[13]

[13] See Supporting Information for experimental details.

[14] Neither Rev-Fl nor the RRE bind to the streptavidin–agarose assembly nonspecifically.[13] Following assembly of the immobilized Rev–RRE complex, addition of excess nucleic acid does not significantly displace Rev-Fl off of the immobilized RRE.[17] In contrast, very low concentrations of the unlabeled Rev peptide or non-biotinylated RRE (40 and 90 nM, respectively) are needed to displace 50% of Rev-Fl. These results taken together suggest a high degree of binding specificity for the immobilized Rev–RRE complex.

[15] Fluorescence anisotropy was used to determine a K_d of 2.3 ± 0.5 nM for Rev-Fl–RRE in solution. A K_d of 2.5 ± 2 nM is measured for the solid-phase-immobilized complex.[13] This K_d value is found to be independent of the "effective loading" of the RRE on the agarose (from 0.1–2.0 pmol mL^{-1} gel), which suggests that the immobilized RRE complexes are noninteracting over this loading range.

[16] Since the binding stoichiometry of these compounds is unknown, their IC_{50} values are reported.

[17] Experiments that include competing nucleic acids contain 15 μg mL^{-1} of plasmid DNA (pGEM) or 65 μg mL^{-1} of a complex mixture of yeast pre-tRNAs and mature tRNAs (Sigma type X). This is approximately a 50-fold molar excess of DNA nucleotides and a 200-fold molar excess of a tRNA nucleotides (relative to the RRE). These concentrations lead to a minimal (5–10%) displacement of the Rev–Fl from the solid support.

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[20] In accordance with the solid phase assay results, gel-shift experiments indicate that all "polycyclic aromatic derivatives" were more effective than neomycin B.[13] $[Ru(bpy)_3]^{2+}$ was also tested by the solid-phase assay, fluorescence anisotropy, and gel shift mobility. Due to fluorescence quenching of Rev–Fl by $[Ru(byp)_3]^{2+}$, fluorescence anisotropy titrations give a false dissociation profile and an IC_{50} value of 170 μM. Both the solid-phase assay and gel shift mobility experiments, however, indicate that $[Ru(bpy)_3]^{2+}$ does not inhibit the Rev–RRE complex at all (up to 10 mM).[13]

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