

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

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Experimental Conditions

Fluorescence anisotropy, solid-phase, and gel shift mobility experiments were conducted at 22°C in a buffer containing 30 mM HEPES (pH 7.5), KCl (100 mM), sodium phosphate (10 mM), NH₄OAc (20 mM), guanidinium HCl (20 mM), MgCl₂ (2 mM), NaCl (20 mM), EDTA (0.5 mM), and Nonidet P-40 (0.001%). This complex mixture of cations and anions is found to minimize the non-specific binding of ligands to the Rev-RRE complex and to maximize the reversibility of the Rev-RRE interaction (as evident in both anisotropy and solid-phase assays). Gel-shift mobility experiments also contained 3% glycerol and .0025% xylene cyanol and bromophenol blue.

Design of Rev Peptides

Both N-terminus and C-terminus modified Rev₃₄₋₅₀ peptides were synthesized and studied. Succinylation of the N-terminus, amidation of the C-terminus, and addition of four alanines has been shown to increase the α helicity of the Rev peptide, and thereby increase its affinity and specificity to the RRE [R. Tan, L. Chen, J.A. Buettner, D. Hudson, A.D. Frankel, *Cell* **1993**, *73*, 1031-1040]. In this case, the alanines also serve as a spacer for subsequent fluorescein modification. The free amine of the "N-terminus modified" peptide is used for conjugation to fluorescein (resulting in "F1-Rev") (see **FigureS1**). The Cys of the "C-terminus modified" peptide is conjugated to fluorescein (resulting in "Rev-F1") and is capped as a thioether acetamide (SCH₂CONH₂) derivative for the non-labeled peptide ("Rev-IA") (see **Figure S1**).

N-terminus modified: NH₂AAAATRQARRNRRRRWRERQR_{am}

C-terminus modified: _{suc} TRQARRNRRRRWRERQRAAAAC_{am}

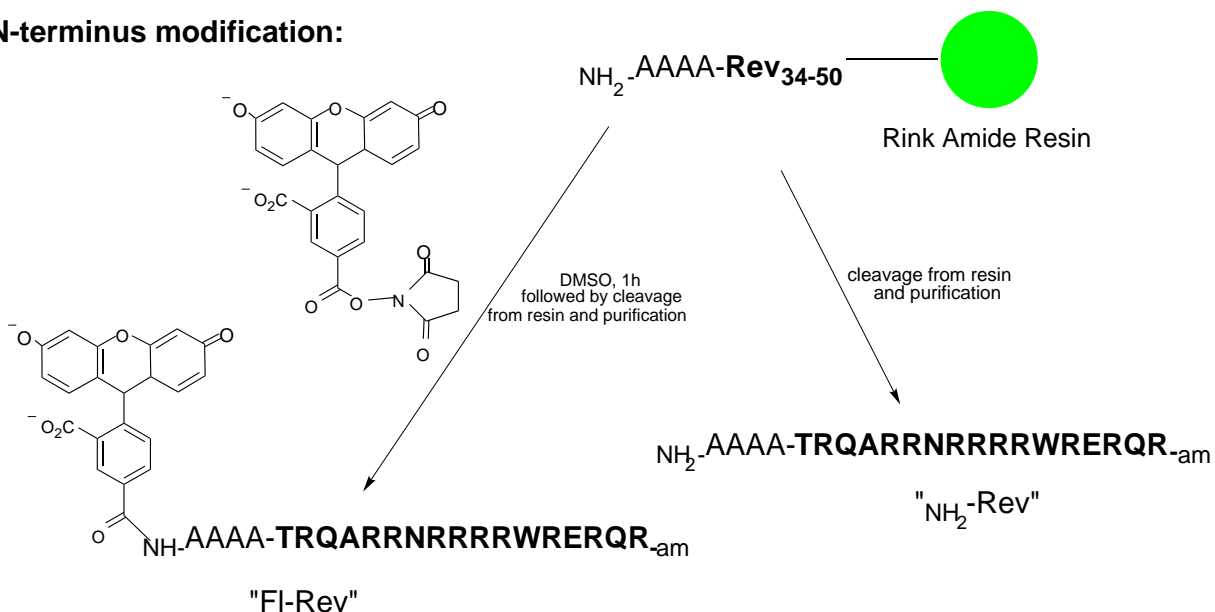
Synthesis of Rev Peptides

Synthesis was carried out using standard Fmoc/HBTU chemistry on ABI Applied Biosystems 431A peptide synthesizer using protected amino acids purchased from Calbiochem. Protected amino acids used in the synthesis were: Fmoc-Arg-(Pbf)-OH, Fmoc-Asn-(Trt)-OH, Fmoc-Gln-(Trt)-OH, Fmoc-Trp-(Boc)-OH, Fmoc-Thr-(tBu)-OH, Fmoc-Glu-(OtBu), and Fmoc-Cys-(Trt)-OH. For the C-terminus modified peptide, the first peptide coupling was done manually to avoid isomerization problems associated with Cys. Fmoc-Cys-(Trt)-OH was converted into its symmetric anhydride by reaction with 0.45 equivalents of DCC in dry methylene chloride for 10 min. The anhydride was separated from the DCU precipitate by vacuum filtration and methylene chloride was subsequently removed in vacuo. The solid anhydride was then dissolved in 30% N-methylpyrrolidone (NMP) in DMF and added to deprotected Rink Amide MBHA resin. The coupling lasted 2.5 hours at 25°C and was monitored by ninhydrin (Kaiser Test). Upon completion, the resin was washed with methylene chloride followed by NMP, and loaded onto the ABI synthesizer. The remainder of the peptide synthesis was carried out using standard HOBt/HBTU chemistry and monitored with a built-in

conductivity meter. For both peptides, the first round of automated coupling reactions was done as a "double coupling" to ensure efficiency. Upon completion of the automated synthesis, the N-terminus modified resin was washed (with methylene chloride) and coupled to fluorescein by reaction with ~100 equivalents of 5-carboxyfluorescein succinimidyl ester (Molecular Probes) in DMSO for 1 h at room temperature (see **Figure S1**). The C-terminus modified resin was washed with DMF and reacted with 0.3 M succinic anhydride, 0.3 M 1-hydroxybenztrazole hydrate, and 0.03 M DMAP in DMF, for 1 h at room temperature. Both reactions were monitored using the Kaiser Test. Upon completion of each reaction, the resin was washed (in methylene chloride) and cleaved from the resin at room temperature for 2.5 h in a "cleavage cocktail" containing 88% TFA, 5% water, 5% phenol, and 2% triisopropylsilane (v/v). The filtrate was then mixed with 15 volumes of 2% acetic acid, and extracted 4 times with diethyl ether. Crude peptides were purified on a C-18 semiprep HPLC column with an isocratic mixture of either 19% or 14% acetonitrile (0.1% TFA) in water (0.1% TFA) (for the fluorescein and non-fluorescein containing peptides respectively). The purified peptides were lyophilized and the C-terminus modified peptide

was reacted with 100 equivalents of 5-iodoacetamidofluorescein in 100 mM sodium phosphate (pH 8.0), 2 mM EDTA, and 30% (v/v) DMSO at room temperature in the dark for 2 h. Or, separately, the purified C-terminus modified peptide was reacted with iodoacetamide (85 mM in 75% DMF/water (v/v) and 20 mM HEPES, pH 8.0) at 25°C in the dark for 2 h (see **Figure S1**). The resulting peptides ("Rev-FI" and "Rev-IA", respectively) were then purified on a C-18 semiprep HPLC column with a mixture of acetonitrile (0.1% TFA) and water (0.1% TFA); isocratic conditions were 19% acetonitrile for Rev-FI and 14% acetonitrile for Rev-IA. Electrospray mass spectrometry confirmed masses for FI-Rev (3,079 amu), NH_2 -Rev (2,721), Rev-FI (3,312 amu), and Rev-IA (2,982 amu). Analytical HPLC confirmed a greater than 98% purity for each peptide. Molecular extinction coefficients were taken as 73,500 $\text{cm}^{-1} \text{M}^{-1}$ for FI-Rev (498 nm), 77,000 $\text{cm}^{-1} \text{M}^{-1}$ for Rev-FI, (498 nm), and 5,600 $\text{cm}^{-1} \text{M}^{-1}$ for both NH_2 -Rev and Rev-IA (280 nm).

N-terminus modification:



C-terminus modifications:

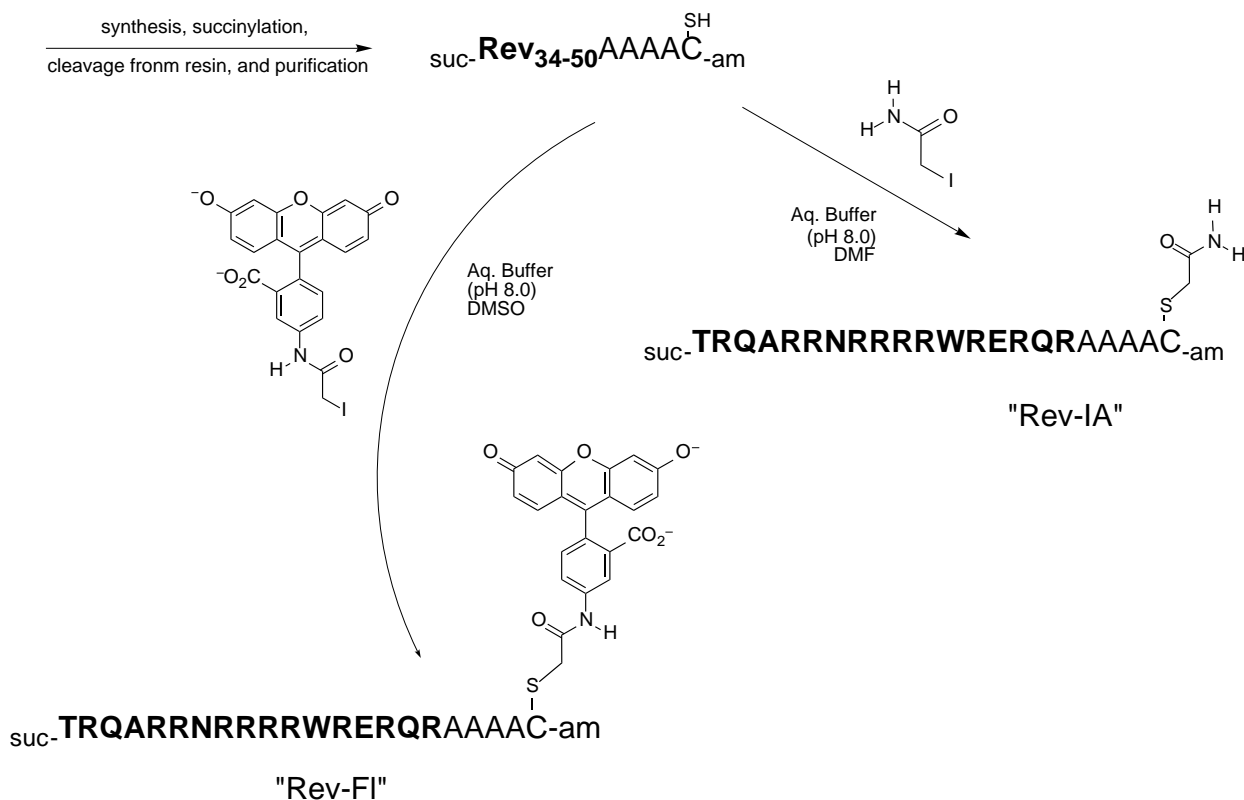


Figure S1. Modification of Rev Peptides.

Evaluation of Rev Peptides

The C-terminus modified peptide is found to be superior to the N-terminus peptide. Gel shift mobility assays indicate that perturbation of RRE affinity is significantly worse when the N-terminus modified peptide is conjugated to fluorescein compared to the addition of fluorescein to the C-terminus modified peptide. Furthermore, upon binding the RRE, fluorescence quenching of "FI-Rev" is much more significant (~30%) than quenching of "Rev-FI" (~10%) (this makes Rev-FI better suited for fluorescence anisotropy experiments). Both observations can be rationalized by examining the solution structure of the Rev-RRE complex which shows the N-terminus of the Rev peptide buried in the major groove of the RRE and the C-terminus free in solution [J.L Battiste, H. Mao, N.S. Rao, R. Tan, D.R. Muhandiram, L.E. Kay, A.D. Frankel, and J.R. Williamson, *Science* **1996**, *273*, 1547-1551].

Synthesis of 67-nt RRE

See **Figure S2** for a flow chart of biotin-RRE synthesis. The synthesis of the 5' biotinylated RRE transcripts is a modification of the procedure described [M. Hendrix, E.S. Priestley, G.F. Joyce, and C. Wong *J. Am. Chem. Soc.* **1997**, *119*, 3641-3648]. To generate RNA transcripts with a phosphorothioate 5'-end, 8 mM guanosine monophosphorothioate (purchased from USB) was included in the transcription reaction (along with 2 mM of each NTP), this approach was found to be more efficient than the previously described method. T7 RNA polymerase was utilized for "run-off" in vitro transcription with a complementary, 83-nt DNA template as described by Uhlenbeck (Uhlenbeck, O.C.; Milligan, J.F. *Methods in Enzymology* **1989**, *180*, 51-62). Synthesis of the 83-nt DNA template was carried out using standard phosphoramidite chemistry. The oligonucleotide was purified using denaturing polyacrylamide gel electrophoresis (PAGE), followed by extraction and multiple rounds of ethanol precipitation. The sequence and homogeneity of the template was confirmed using di-deoxy sequencing techniques. Transcription products were also purified using denaturing PAGE, extraction and multiple rounds of ethanol precipitation. Transcripts with a phosphorothioate 5' end were then reacted with Iodoacetyl-LC-Biotin (purchased from Pierce) as described [M. Hendrix, E.S. Priestley, G.F. Joyce, and C. Wong *J. Am. Chem. Soc.* **1997**, *119*, 3641-3648] and re-purified as above. The expected sequence of the 67-nt RNA transcript was verified by 5'-end labeling a RNA transcript (having a 5' hydroxyl) with ^{32}P and subsequent enzymatic digestion (RNase T1, A, and U2). The molecular extinction coefficient for the biotinylated and non-biotinylated 67-nt RNA transcript was taken as $741,400 \text{ cm}^{-1} \text{ M}^{-1}$ (at 260 nm). Before use, RNA transcripts were folded in a buffer containing 30 mM HEPES (pH 7.5), KCl (100 mM), MgCl_2 (2 mM), and EDTA (0.5 mM) by heating to 90°C and cooling to room temperature over 30 min.

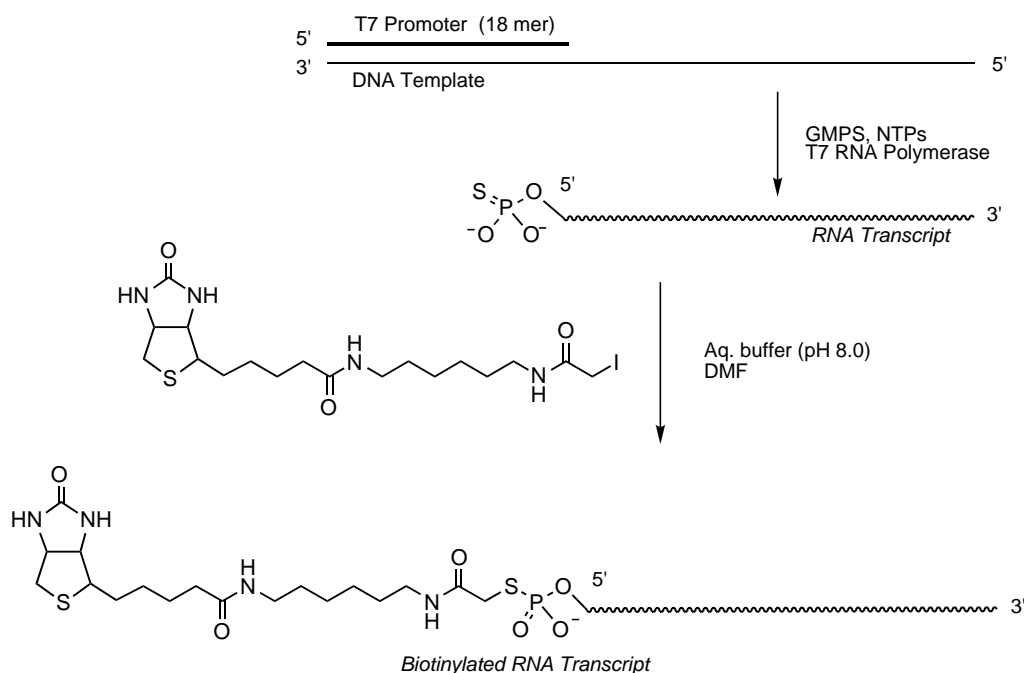


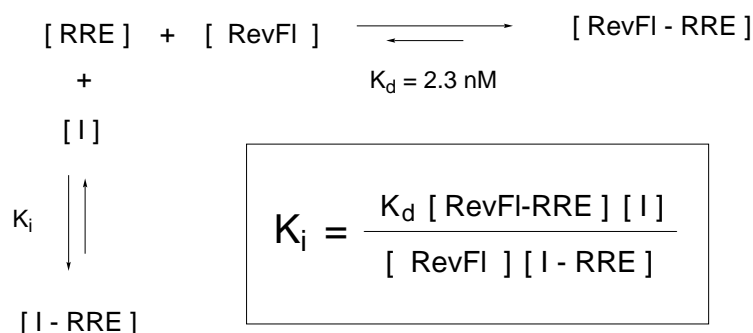
Figure S2. Scheme of biotin-RRE synthesis.

Fluorescence Anisotropy

See **Figure S3 (a)** and **(b)** for anisotropy data showing RevFl-RRE association and dissociation.

For dissociation experiments, a Rev-RRE complex was formed by mixing 10 nM Rev-Fl with 100 nM of the pre-folded 67-nt RRE in a thermocontrolled (22°C) cuvette. Inhibitors were then titrated and both total fluorescence and anisotropy were monitored (Perkin Elmer LS-50B fluorimeter, maximum slit widths, Excitation 490 nm, Emission 530 nm, 2 second integration time, 6 readings averaged per concentration). The K_d of the RevFl-RRE interaction is calculated (**Figure S3(a)**) by titration of the prefolded 67-nt RRE fragment into a solution of 10 nM Rev-Fl (See: T. Heyduk, J.C. Lee, *Proc. Natl. Acad. Sci. USA* **1990**, 87, 1744-1748. F.W. Sevenich, J. Langowski, V. Weiss, K. Rippe, *Nucleic Acids Res.* **1998**, 26, 1373-1381).

The K_d obtained for Rev-Fl binding to the RRE was used to calculate a K_i value for Rev-IA. Upon binding the RRE, only minor changes in the emission spectrum of fluorescein were seen (about 10% quenching of Rev-Fl). We have taken the change in anisotropy as being directly proportional to the fraction of Rev-Fl bound by the RRE. Sufficient mixing time was always provided to allow for equilibrium to be reached. From a simple, three component, competitive binding equilibrium, the following equation can be derived:



By this analysis, a K_i of 1.2 ± 0.8 nM is calculated for Rev-IA, which compares to a K_d of 2.3 ± 0.5 nM for Rev-Fl. This shows that addition of fluorescein has a only minor effect on RRE affinity. Since binding stoichiometries of the other inhibitors are unknown, IC_{50} values (inhibitor concentrations which give 50% displacement of RevFl) are reported.

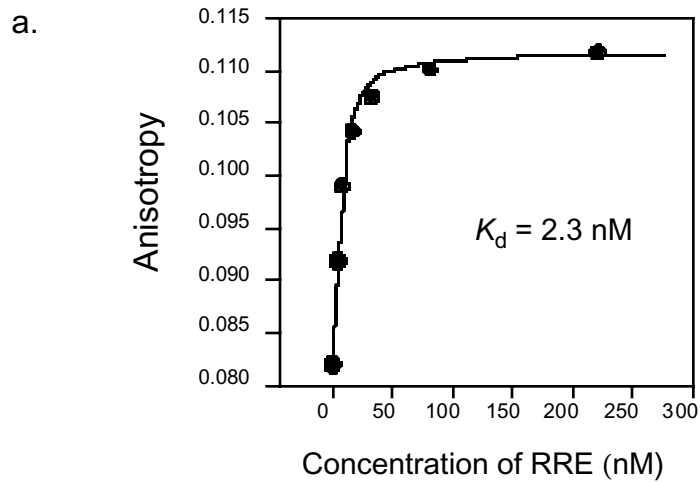


Figure S3a. Both line shape and gel shift analysis indicate a 1:1 complex of the RRE to Rev-FI. Given this, a K_d of 2.3 nM was calculated by nonlinear regression using the equation:

$$A = A_0 + \Delta A \frac{([\text{RNA}]_{\text{total}} + [\text{Rev-FI}]_{\text{total}} + K_d) - \sqrt{([\text{RNA}]_{\text{total}} + [\text{Rev-FI}]_{\text{total}} + K_d)^2 - 4[\text{RNA}]_{\text{total}} [\text{Rev-FI}]_{\text{total}}}}{2[\text{Rev-FI}]_{\text{total}}}$$

A= anisotropy of the Rev-FI

A_0 = anisotropy of the Rev-FI in the absence of RNA

ΔA =the total change in anisotropy at saturation of the Rev-FI

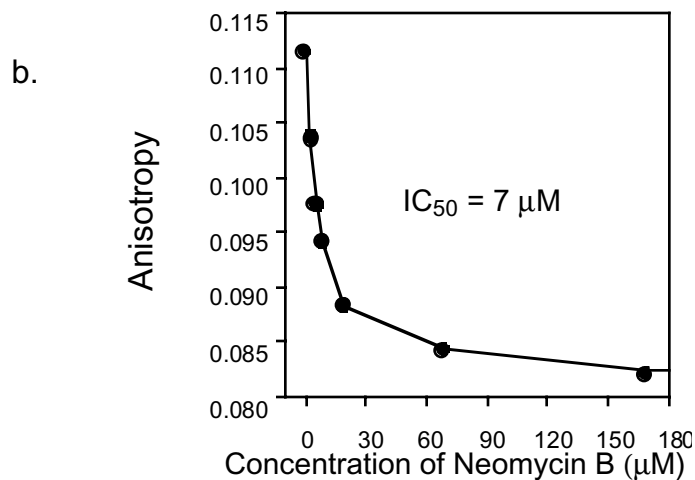


Figure S3b. Example of a fluorescence anisotropy displacement experiment, where the Rev-RRE complex is disrupted with increasing concentration of inhibitor.

Assembly of the Solid-Phase Immobilized Rev-RRE Complex

“ImmunoPure” beaded agarose covalently modified with streptavidin was purchased from Pierce. Immediately before use, the resin is washed with 3 volumes of buffer (under “Experimental Conditions”) with gentle mixing (by inversion of the tube). After each wash (4 total) the slurry of solid-support and buffer is gently centrifuged (1,000 rpm 30 s) to settle the agarose beads, and the buffer removed by pipette. Following the last wash, 500 μL of dilute biotinylated RRE (400 nM in buffer) is added to 1 mL of a 40% slurry (400 μL of beads and 600 μL of buffer) and immediately inverted to mix. The slurry is incubated at room temperature for 1.5 h with constant inversion for mixing. By monitoring absorbance of the supernatant at 260 nm, the efficiency of RRE immobilization is calculated. The loading efficiency is >95% for biotinylated RRE and is <1% for non-biotinylated RRE. Following RRE immobilization, 1 equivalent of Rev-FI is added, incubated for 1 h, and the supernatant quantified for fluorescence intensity. Under these conditions, non-specific binding of Rev-FI to the solid-support is not observed (as determined by addition of RevFI to streptavidin-agarose which lacks immobilized RRE). Since a linear relationship between fluorescence intensity and concentration of Rev-FI is established by a calibration of UV absorbance and fluorescence emission, the concentration of Rev-FI in the supernatant (following incubation with the immobilized RRE) can be calculated by its fluorescence intensity. It is found that under the above loading conditions, ~85% of Rev-FI is bound by the immobilized RRE. The K_d of the RevFI-RRE interaction on solid support is then calculated by assuming that 85% of the RRE is bound by Rev-FI (which is confirmed by mixing a known volume of gel with 8M guanidinium HCl and quantifying the amount of Rev-FI present on the gel itself). The effective “surface concentration” of the RRE is irrelevant in this special circumstance, since the surface volume of free RRE cancels with the surface volume of Rev-RRE complex:

$$K_d = [\text{RRE}][\text{Rev-FI}]/[\text{complex}] = (\text{moles of free RRE})[\text{Rev-FI}] / (\text{moles of complex})$$

A K_d of 2.5 +/- 2 nM is measured, and is found to be independent of the “effective loading” of the RRE on the agarose (from 0.1 pmoles RRE/ μL gel through 2.0 pmoles RRE/ μL gel), suggesting that the immobilized RRE complexes are non-interacting over this loading range.

Conducting the Solid-Phase Assay

In 0.75 mL siliconized tubes, 200 μL of buffer (see “Experimental Conditions”) is added, followed by 30 μL of a 30% Rev-RRE slurry (at 0.5 pmole RRE/ μL of gel). A small volume of concentrated inhibitor and/or compeditor is added, mixed gently, and incubated at room temperature for 90 minutes with constant inversion for mixing. The tube is then gently centrifuged (1,000 rpm for 30 s) to settle the beads. All 220 μL of supernatant is then added to 780 μL of “quantification buffer” (4M guanidinium HCl, 125 mM Tris HCl (pH 9.0), 125 mM KCl, 25 mM MgCl_2 , 0.005% Nonidet P-

40), and quantified for fluorescence intensity (Perkin Elmer LS-50B fluorimeter, maximum slit widths, Excitation 490 nm). Controls which establish the minimum (no inhibitor added) and maximum signals (200 μ L of 8M guanidinium HCl added instead of buffer) are done with each set of experiments. See **Figure S4** for an example titration.

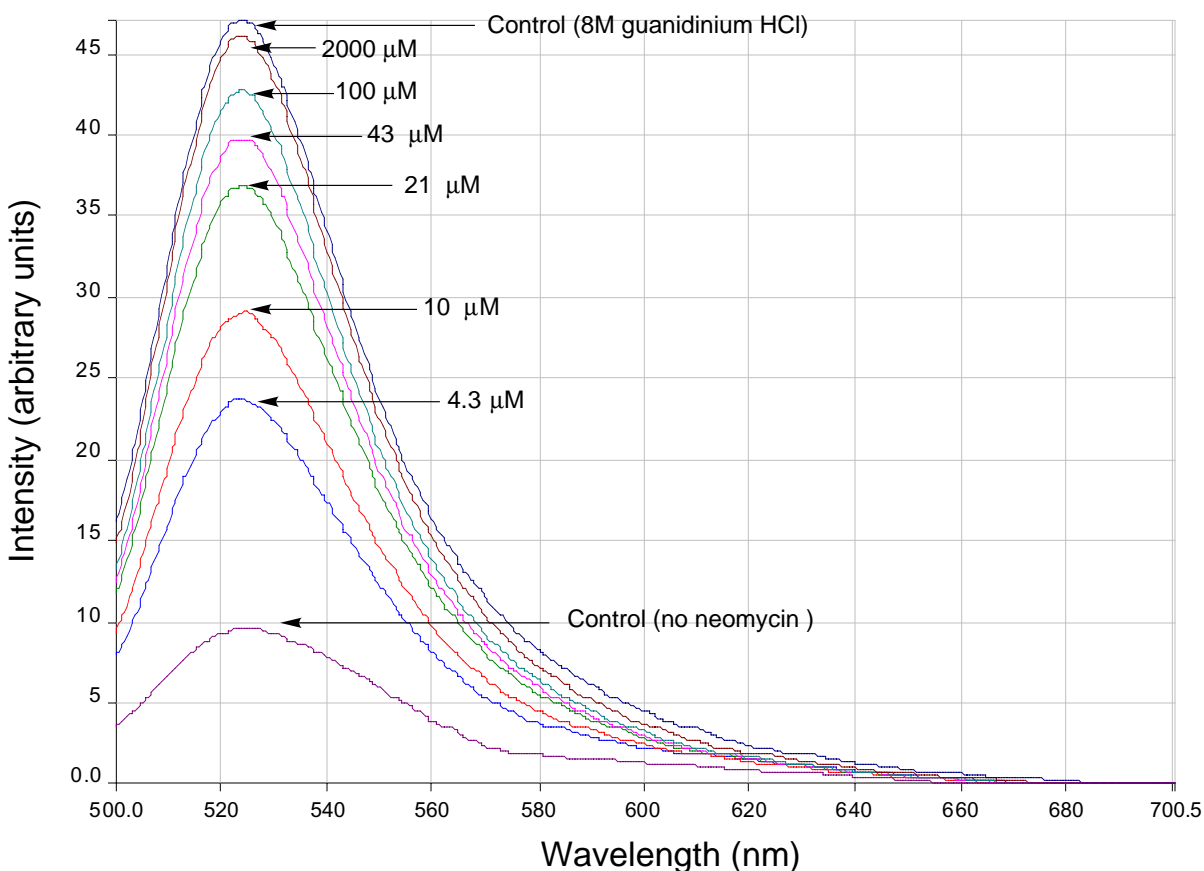


Figure S4. Raw data from solid-phase displacement experiments shows the fluorescence emission of the supernatant as a function of neomycin B concentration. To reduce light scattering at 500 nm, excitation of Rev-FI is at 470nm. Excitation at 490nm also yields the same IC_{50} values.

For inhibitors which interfere with emission of Rev-FI, the quantity of Rev-FI remaining on the agarose beads is quantified by removing the supernatant (as above) then washing the beads 2 times with 200 μ L of isopropanol. After each wash the mixture is gently centrifuged (1,000 rpm, 30 s) to settle the agarose beads, and isopropanol removed by pipette. 500 μ L of 8M guanidinium HCl is then added to the beads, mixed, heated to 55 $^{\circ}$ C, mixed again, then centrifuged to settle the agarose beads. All 500 μ L of supernatant is then added to 500 μ L of “quantification buffer” (see above) and quantified for fluorescence intensity (as above).

Gel Shift Experiments

To address the discrepancies between IC_{50} values (in Table 1 (manuscript)) derived from the solid-phase assay and those from fluorescence anisotropy, gel shift experiments were conducted to test the inhibitory activity of neomycin B relative to the “aromatic diamidines”. A Rev-RRE complex was formed by mixing 10 nM RRE (a fraction 5'-end labeled with ^{32}P) with 80 nM “Rev-IA”(see above) in the buffer described under “Experimental Conditions”. This mixture was incubated at room temperature for 15 min then 9 μ L aliquots were added to tubes containing 1 μ L of a inhibitor solution, the mixture was incubated for an additional 15 min and loaded onto a native 17% polyacryamide gel. The running buffer and gel (1:29 bis : acrylamide) were at 1X TBE. The gel was run overnight at a constant 300 V and exposed to a Molecular Dynamics Phosphor Screen and PhosphorimagerTM 445 SI and analyzed with ImagequantTM software (Molecular Dynamics), see **Figure S5**. The results indicate that the inhibitory trends exhibited by the solid phase assay are reproduced in the gel shift mobility experiment.

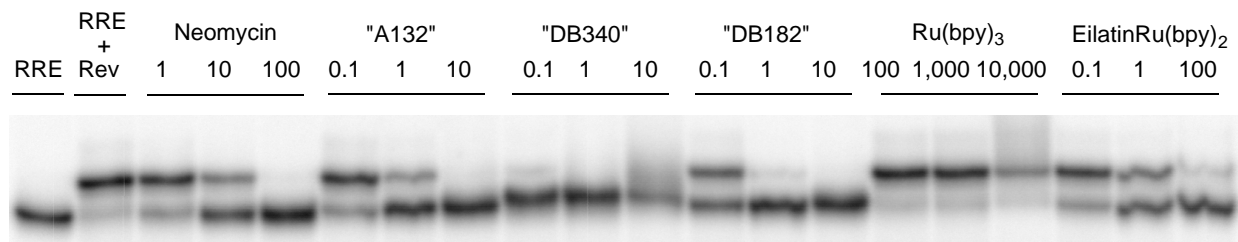


Figure S5. In accordance with the solid phase assay, gel shift analysis indicates the following inhibitory trend: DB340 > DB182 > A132 > Eilatin Ru (bpy)₂ > Neomycin B >>> Ru(bpy)₃. Indicated concentrations are in μ M.