Eilatin Ru(II) Complexes Display Anti-HIV Activity and Enantiomeric Diversity in the Binding of RNA

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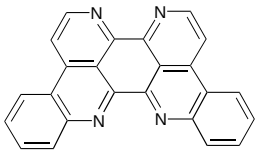
Eilatin-containing octahedral ruthenium complexes inhibit HIV-1 replication in CD4+ HeLa cells and in human peripheral blood monocytes with IC_{50} values of approximately 1 μM. Similar metal complexes that lack eilatin display 15–100-fold lower anti-HIV activities. [Ru(bpy)$_2$"pre-eilatin"]$^{2+}$, a complex that contains a nonplanar analogue of eilatin, shows significantly lower nucleic acid binding and lower anti-HIV activity than eilatin complexes. This result indicates that the extended planar surface presented by eilatin is important for both activities. Rev peptide and ethidium bromide displacement assays are used to probe the nucleic acid affinity and specificity of Λ- and Δ-[Ru(bpy)$_2$eilatin]$^{2+}$. Two HIV-1 RNA sites are compared and a significant binding preference for the Rev response element over the transactivation response region is found. Simple DNA duplexes show a consistent selectivity for Λ-[Ru(bpy)$_2$eilatin]$^{2+}$ compared to Δ-[Ru(bpy)$_2$eilatin]$^{2+}$, while RNAs show more diverse enantiomeric selectivities.

KEYWORDS:

DNA recognition · HIV · medicinal chemistry · RNA recognition · ruthenium

Introduction

Eilatin (**1**) is a fused, heptacyclic aromatic alkaloid that was isolated from the Red Sea tunicate *Eudistoma sp*.[1] It is reported to possess cytotoxic and antiproliferation

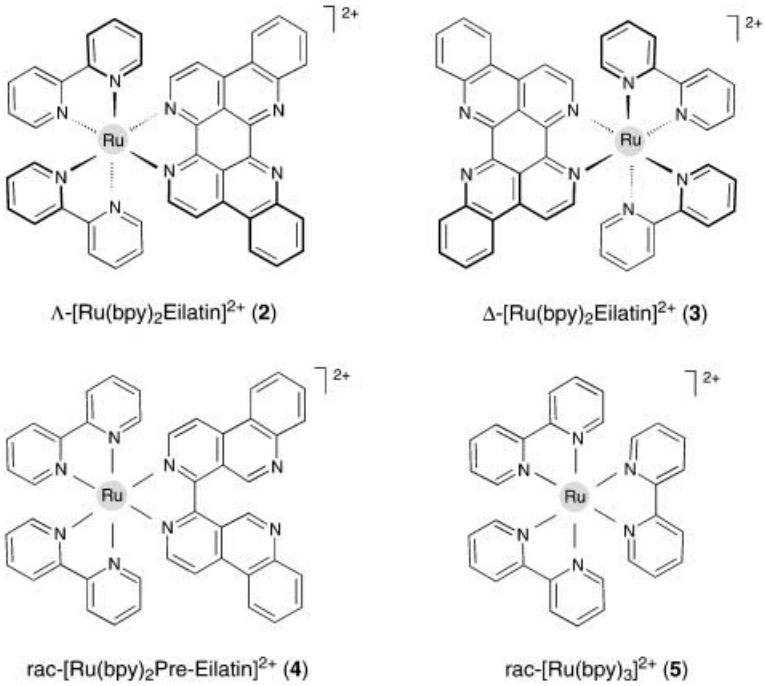


Eilatin (**1**)

activities in a broad range of tissue cultures.[2–4] The antitumor activity of eilatin and its planar polycyclic structure, lead Ireland to examine its ability to bind DNA. Ethidium bromide displacement and other fluorescence experiments suggested, however, that eilatin (**1**) binds to DNA with low affinity (IC_{50} for ethidium displacement >100 μM).[4, 5]

Eilatin is potentially a bifacial metal chelator. Upon incorporation into octahedral metal complexes of the type [Ru(L)$_2$eilatin]$^{2+}$ (where L = 2,2′-bipyridine (bpy), phenanthroline (phen), etc.), only the less hindered face of eilatin binds to the metal ion (Scheme 1).[6] Since numerous octahedral metal complexes are known to bind nucleic acids,[7] we were intrigued by the potential use of eilatin-containing metal complexes to selectively target unique RNA structures within the genome of HIV-1.

Association of the Rev response element (RRE) and of the transactivation response region (TAR) with their biological



Scheme 1. *Octahedral metal complexes used in these studies. Unlike "free" eilatin (**1**), the dichloride salts of **2**–**5** are readily soluble in water. rac = racemic.*

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ligands (Rev and Tat proteins, respectively) are events necessary for the replication of HIV-1.[8, 9] The Rev and Tat proteins contain homologous arginine-rich sequences that serve as the RNA binding domain for each protein (Figure 1). Inhibition of these protein – RNA interactions may lead to therapeutic agents that inhibit viral replication by binding to an RNA regulatory element.[10, 11] The enantiomerically pure metal complexes Λ-[Ru(bpy)$_2$eilatin]$^{2+}$ (**2**) and Δ-[Ru(bpy)$_2$eilatin]$^{2+}$ (**3**) were previously found to bind to the RRE with high affinity and high specificity (relative to a mixture of tRNAs).[12] A solid-phase fluorescent binding assay and native gel-shift electrophoresis showed that the eilatin-containing complexes **2** and **3** bind the RRE and displace a Rev protein fragment with approximately five times greater activity than the widely studied RNA ligand neomycin B.[12] The solid-phase assay also indicated that both **2** and **3** have a significant affinity for double-stranded DNA and that DNA binds **2** in preference to **3**.[12]

In this report, we disclose the anti-HIV activity of **2** and **3** and compare their activities to those of two structural analogues, [Ru(bpy)$_2$"pre-eilatin"]$^{2+}$ (**4**) and [Ru(bpy)$_3$]$^{2+}$ (**5**). A correlation is found between the RRE affinity and the anti-HIV activity of these compounds. Other nucleic acids (calf thymus (C. T.) DNA, an HIV-1 TAR RNA construct, and simple polymeric DNAs and RNAs) were also evaluated for their affinity for **2**, **3**, and **4**. Ethidium displacement experiments indicate that **2** and **3** have a higher affinity for all nucleic acids as compared to **4**. Thermal denaturation experiments support the trends observed in the fluorescent displacement assays and confirm the consistent selectivity of DNA for **2** over **3**. RNAs, however, are found to exhibit more diverse enantiomeric selectivities.

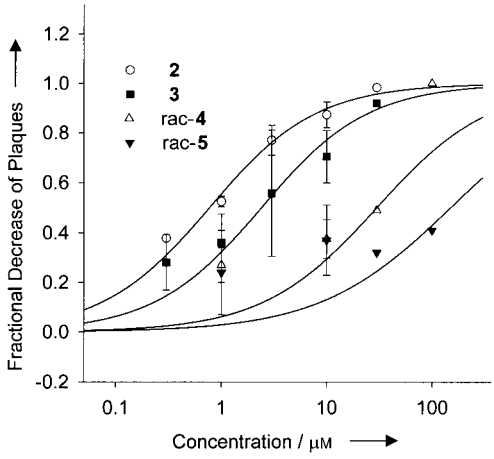

Figure 2. *Anti HIV-1 activities of **2** – **5** as evidenced by the fractional decrease in plaque-forming units.*

Table 1. *IC_{50} values for inhibition of HIV-1 and peptide displacement.*

Compound	HIV-1[a]	RRE67[b]	TAR31[b]
2	0.8	0.9	5.3
rac-**2**/**3**	0.9	1.0	3.9
3	2.0	1.1	3.5
rac-**4**	30	20	30
rac-**5**	>100	>170[c]	>86[c]

[a] Concentration (μM) needed to decrease HIV-1 activity by 50% in a HeLa plaque assay. The standard deviation is less than $\pm$40% of the reported values.[13] [b] Concentration (μM) needed to displace 50% of Rev-Fl from 100 nM of RNA construct. The standard deviation is less than $\pm$20% of the reported values. [c] Only limits can be determined with this method as a result of fluorescence interference from [Ru(bpy)$_3$]$^{2+}$.[12]

Results and Discussion

Anti-HIV activity

The anti-HIV activities of compounds **2** – **5** were measured in HIV-1 infected CD4+ HeLa cell cultures by using a plaque-formation assay.[13, 14] The dose-dependent decrease in plaques (syncytia) is shown in Figure 2 and IC_{50} values are summarized in Table 1. Compounds **2** and **3** have anti-HIV activities that are from 15 to over 100-fold greater than those of **4** and **5**. This clearly illustrates the significance of the eilatin moiety for the anti-HIV activity of **2** and **3**.[15] To date, only one other family of ruthenium-containing complexes has been shown to inhibit HIV replication.[16] However, these compounds are chemically reactive, create covalent cross links with DNA, and are reported to be highly toxic.[16] In contrast, compounds **2** – **5** are chemically inert, and show no sign of toxicity to HeLa cells (up to 100 μM).

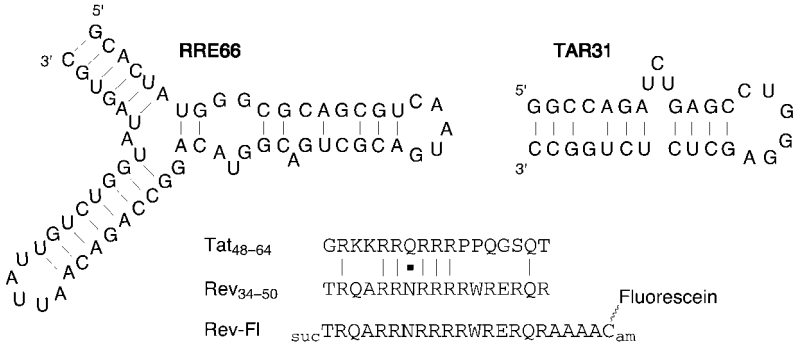

Figure 1. *The minimal binding domains of the RRE and TAR used in these studies.[35, 36] Sequence homology between the arginine-rich domains of Rev_{34-50} and Tat_{48-57} and the fluorescent peptide Rev-Fl is shown. suc = succinylated; am = amidated.*

Binding of RRE and TAR

To investigate the relative affinities of **2** – **5** for the HIV-1 RRE and TAR, fluorescence anisotropy was used to monitor the ability of each compound to displace a fluorescent arginine-rich peptide from each RNA. Both RNAs have been found to bind the Rev_{34-50} protein fragment with 1:1 stoichiometry.[17] The association of each RNA with a fluorescent Rev_{34-50} protein fragment (Rev-Fl) is evident from the increased fluorescence anisotropy upon titration of either TAR or RRE (Figure 3a). Nonlinear curve fitting yields a dissociation constant K_d of 2.3 $\pm$ 0.5 nM for the affinity of Rev-Fl for RRE66 and 21 $\pm$ 8 nM for Rev-Fl

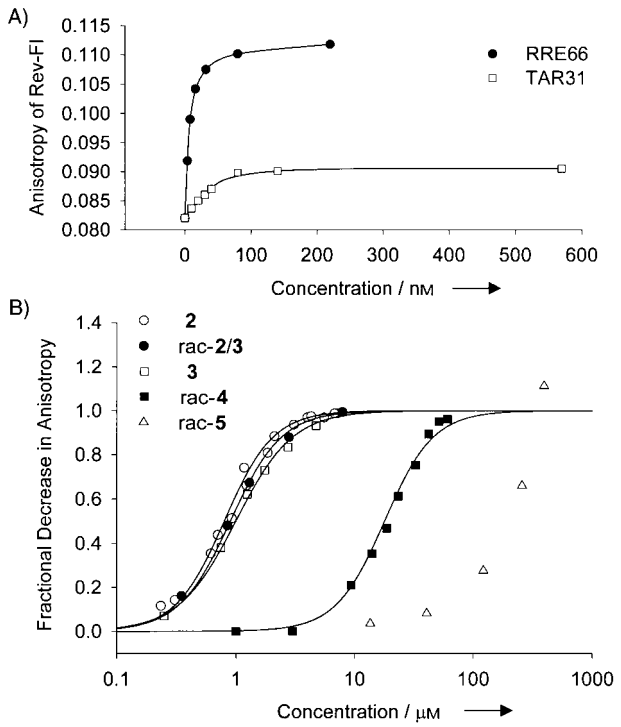

Figure 3. *Examples of peptide binding and subsequent displacement experiments. A) Association of Rev-Fl to either RRE66 or TAR31, as evidenced by the increased fluorescence anisotropy of Rev-Fl. Note that the relative size of each RNA construct is reflected in the magnitude of the change in anisotropy at saturation. B) Representative Rev-Fl displacement isotherms. A fractional change in anisotropy of 1.0 indicates a return of Rev-Fl to its anisotropy value when free in solution (0.081). Notice how $[Ru(bpy)_3]^{2+}$ (**5**) causes a decrease past this value while the other inhibitors reach saturation at 1.0. **5** has been proven to interfere with this assay and does not inhibit Rev – RRE binding up to 10 mM.[12].*

binding to TAR31.[18] Upon complex formation, inhibitors were added and the displacement of Rev-Fl was observed as a decrease in anisotropy. Examples of displacement experiments are shown in Figure 3b and the IC_{50} values of compounds **2** – **5** are summarized in Table 1.

The concentrations of compounds **2** – **5** needed for 50% Rev-Fl displacement correlate with the IC_{50} values for inhibition of HIV-1 replication (Table 1). Compounds **2** and **3** are significantly more effective for Rev-Fl displacement than compounds **4** and **5**. Preferential binding of both **2** and **3** to RRE66 relative to TAR31 is indicated by the three- fivefold lower IC_{50} values observed for peptide displacement from the RRE.[19] Opposite trends in enantiomeric selectivity for each RNA are also observed. The TAR31 construct shows a small enantiomeric selectivity for **3** over **2**, while the RRE shows a slight preference for **2** over **3**.[20] Compared to both **2** and **3**, the nonplanar $[Ru(bpy)_2$"pre-eilatin"$]^{2+}$ analogue (**4**) has significantly diminished RRE affinity and anti-HIV activity. Crystal structures of **2** and **3** show eilatin to be nearly planar.[21] The absence of a single carbon – carbon bond in $[Ru(bpy)_2$"pre-eilatin"$]^{2+}$ (**4**) should impart a fluctuating dihedral twist that averages 25° between the two fused tricyclic aromatic systems.[22] Prior to these studies, it was unknown how this twist would affect nucleic acid affinity.[23] The inability of $[Ru(bpy)_3]^{2+}$ (**5**) to bind to nucleic acids appears to be a general phenomenon.[7f] This indicates that complementary electrostatic interactions are not the primary energetic driving force for the binding of these metal complexes to nucleic acids.

Ethidium displacement from seven nucleic acids

Ethidium bromide displacement assays were conducted to confirm the trends observed by peptide displacement experiments and to expand the study to include simple duplex RNAs and DNAs. Figure 4 depicts typical data from these experiments.

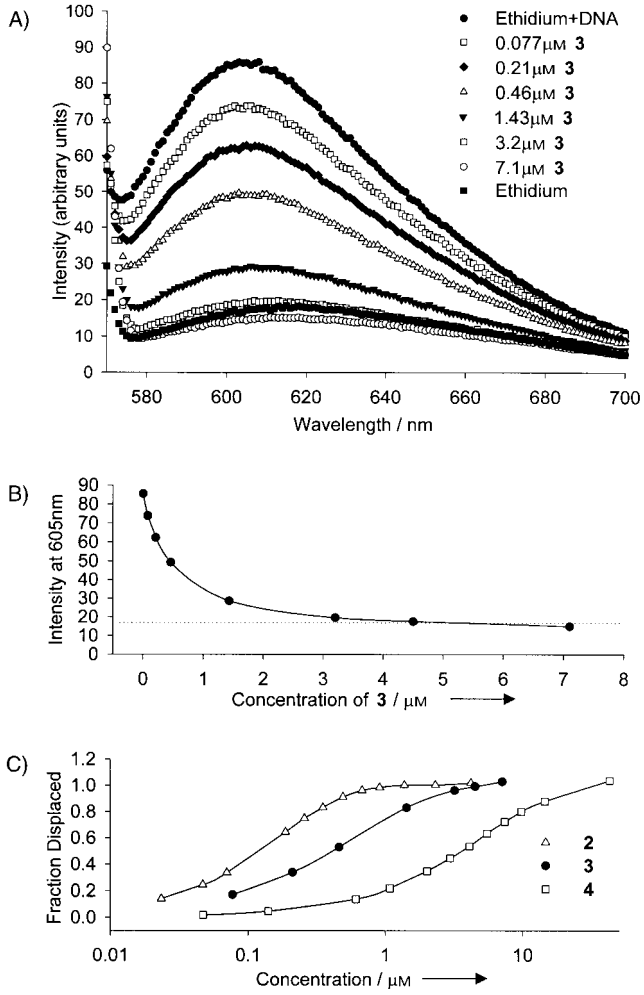

Figure 4. *Representative examples of ethidium bromide displacement assays. A) Raw emission data for 1.25 μM ethidium bromide upon excitation at 546 nm in buffer only (black square) and upon addition of 0.88 μM base pairs C.T. DNA (black circles). **3** was then titrated from 0.077 μM to 7.1 μM. B) The decrease of fluorescence intensity of the ethidium – DNA complex with increasing concentrations of **3**. The dotted line indicates the fluorescence intensity of 1.25 μM free ethidium in buffer only. C) Classic S-shaped binding isotherms are obtained by assuming a linear relationship between the change in fluorescence intensity and the fraction of ethidium displaced. This allows for the determination of the concentration of each inhibitor needed to displace half of the ethidium from C.T. DNA (summarized in Table 2). A small amount of direct quenching of free ethidium by all the metal complexes is evident in the secondary linear element of each isotherm, which becomes apparent from around 5 μM. This direct quenching of ethidium results in final intensities that are lower than that of free ethidium and serves to artificially increase the apparent activity of all inhibitors with IC_{50} values greater than about 3 μM.*

Table 2. *Ethidium displacement IC_{50} values.*[a]

Compound	RRE66[b]	TAR31[b]	r(I) – r(C)[c]	r(I) – r(C)[d]	r(A) – r(U)[d]	C.T. DNA[d]	poly [d(AT)][d]	poly [d(GC)][d]
2	0.25	1.8	10.5	1.5	2.0	0.1	0.07	0.25
3	0.4	1.6	7.7	1.4	5.0	0.4	0.2	0.4
rac-**4**	10	9.0	22	4.0	23	7.0	n.d.[e]	n.d.[e]

[a] Values are in μM. Errors associated with each measurement are within ± 20% of the reported value. [b] IC_{50} values measured by using 50 nM of RNA construct. [c] IC_{50} values measured by using 11 μM of duplex base pairs. [d] IC_{50} values measured by using 0.88 μM of duplex base pairs. [e] Not determined.

Table 2 summarizes the IC_{50} values of **2**, **3**, and **4** for ethidium displacement from RRE66, TAR31, poly [r(I)] – poly [r(C)] duplex RNA, poly [r(A)] – poly [r(U)] duplex RNA, C.T. DNA, poly [d(AT)] – poly [d(AT)] duplex DNA, and poly [d(GC)] – poly [d(GC)] duplex DNA.[24] Even though ethidium bromide is regarded as a nonspecific intercalating agent,[25] it has been shown to bind to simple duplexes with variable stoichiometries and a broad range of affinities.[26] The IC_{50} values in Table 2 are, therefore, not comparable between different nucleic acids.[27]

Ethidium displacement experiments indicate that, in general, **2** and **3** have a higher affinity for all nucleic acids as compared to **4**. These experiments also indicate that RRE66 exhibits preferential binding of **2** over **3**, while the TAR31 has the opposite selectivity. These results are consistent with Rev-FI displacement experiments.[28] Simple duplex RNAs also show complex enantiomeric selectivity. Poly [r(A)] – poly [r(U)] shows selectivity for **2** over **3**, while poly [r(I)] – poly [r(C)] shows a small yet consistent preference for **3** over **2** (Table 2). All three of the simple duplex DNAs show preferential binding of **2** over **3**. Interestingly, this is the opposite enantiomeric selectivity as compared to most other metal-complex – DNA interactions reported to date.[7a, 7c–f]

In our experience, fluorescent displacement assays can be prone to artifacts (especially for evaluation of ligands that are themselves emissive or can directly quench the fluorescence of the displaced species).[12] For this reason, we used thermal denaturation of duplex C.T. DNA as an independent method to confirm the trends observed by ethidium displacement. Compared to C.T. DNA alone, a negligible increase in the melting temperature (T_m) is observed upon addition of **4** ($\Delta T_m = +0.3 \pm 0.3\,^{\circ}C$), a larger increase in T_m is seen with **3** ($\Delta T_m = +4.3 \pm 0.3\,^{\circ}C$), and the largest increase is observed with **2** ($\Delta T_m > +9.1\,^{\circ}C$).[29] These results correlate with the apparent affinity of each compound for C.T. DNA, as indicated by ethidium displacement measurements. Thermal denaturation of RNAs also confirms that **4** shows only very weak stabilization of duplex RNA, while **2** and **3** show more dramatic effects (data not shown).

Conclusions

The eilatin-containing metal complexes **2** and **3** are found to have significant anti-HIV activities in cell cultures.[13] Evaluation of the structural analogue **4** indicates that the planarity of the eilatin moiety within **2** and **3** is essential for both the anti-HIV activity and the nucleic acid affinity of these complexes.[30] For all complexes evaluated, the trends for Rev – RRE inhibition are similar to those for HIV-1 inhibition. This correlation provides some evidence that interference with Rev – RRE activity is the mechanism responsible for the anti-HIV activity of **2** and **3**. The nucleic acid enantiomeric selectivity for **2** and **3** is complex. All DNA duplexes evaluated thus far have a clear preference for binding of **2** over **3**, while RNA shows more diverse behavior. The RRE shows a slight preferential binding with **2** over **3**, while the TAR shows a small preferential binding of **3** over **2**. Poly [r(A)] – Poly [r(U)] shows preferential binding of **2** over **3**, while Poly [r(I)] – Poly [r(C)] shows the opposite selectivity. To our knowledge, **2** and **3** are the first examples of octahedral metal complexes to exhibit variable chiral discrimination between simple duplex RNAs.[31]

Materials and Methods

Metal complexes: The dichloride salts of **2** – **5** were used for all experiments. The synthesis and characterization of rac-**2**/**3** has been reported previously.[6] Complexes **2** and **3** were synthesized from enantiomerically pure precursors.[32] To confirm the assigned absolute configuration of **2** and **3** as Λ and Δ respectively, the CD spectrum of each was recorded.[29] The dominant transitions (between 260 and 300 nm) match those predicted by exciton theory for the correct assignment of the absolute configuration.[33] The synthesis and full characterization of rac-**4** will be reported elsewhere. ESI MS: calcd for $C_{44}H_{30}F_6N_8PRu$ (**4**): 917 $[M]^+$; found: 917 (with predicted isotopic distribution pattern); UV/Vis for **4** in CH_3CN: λ_{max}/nm ($\varepsilon \times 10^{-3}$) = 242 (58), 286 (73), 357 (24), 397 (13), 539 (8.5). These extinction coefficients are very similar to those measured for rac-**2**/**3**.[6] HPLC analysis (C18 reversed phase, monitored at 260 nm) indicated a greater than 95% purity of all metal complexes tested.

HeLa plaque assay: Two independent sets of duplicate points were collected as previously described.[14] HT-6C cells were grown and assayed for plaque-forming units (PFUs), in Dulbecco's Modified Eagle's Medium that contained fetal calf serum (10%), glutamine (2 mM), and penicillin and streptomycin (100 $\mu g\,mL^{-1}$ each). Cells were seeded in 24-well Falcon plates at 2.5×10^4 cells/well and incubated overnight at 37 °C in the presence of CO_2. The HIV-1 strain LAI X794 was then added such that 70 ± 10 PFUs/well were apparent after an additional 3-day incubation. Inhibitors were added 2 h after the addition of HIV-1 and incubated as above for 3 days. Cells were then washed in MeOH and stained with Crystal Violet (0.5% in H_2O). Cell density and PFUs were counted and compared to controls with no inhibitor present. As a control, AZT was included in each round of testing and consistently showed an IC_{50} value of approximately 0.01 μM.

Nucleic acids: RRE66 and TAR31 were transcribed by using T7 RNA polymerase and a DNA template as described previously.[34] The accuracy and homogeneity of the synthetic DNA templates were confirmed by automated dideoxy sequencing. Transcribed RNAs were purified by denaturing polyacrylamide gel electrophoresis

followed by extraction and two rounds of ethanol precipitation. The expected masses of the TAR and RRE were confirmed by using MALDI-TOF mass spectrometry. The UV absorbance at 260 nm (pH 7.5) was used to quantify the nucleic acids (ε_{RRE} = 741 400 $cm^{-1}M^{-1}$ and ε_{TAR} = 323 900 $cm^{-1}M^{-1}$) after base hydrolysis (1 M NaOH, 90 °C, 10 min, quenched with 1 M HCl). Sonicated C. T. DNA was purchased in solution from Gibco BRL and quantified (in duplex form) by measurement of the UV absorbance (ε_{260nm} = 13 100 $cm^{-1}M^{-1}$ base $pair^{-1}$). The remaining RNA and DNA duplexes were purchased from Pharmacia, suspended in 1X TE and quantified (in duplex form) by using the reported UV extinction coefficients.[26]

Nucleic acid binding conditions: All displacement and T_m experiments were conducted in a buffer that contained 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (30 mM; pH 7.5), KCl (100 mM), sodium phosphate (10 mM), NH_4OAc (20 mM), guanidinium HCl (20 mM), $MgCl_2$ (2 mM), NaCl (20 mM), ethylenediaminetetraacetate (0.5 mM), and Nonidet P-40 (0.001 %). This complex mixture of salts is found to minimize the nonspecific binding of ligands to the Rev – RRE complex and to maximize the reversibility of the Rev – RRE interaction.

Peptide displacement: The synthesis and characterization of Rev-Fl is reported in the supplementary information of reference [12]. Fluorescence anisotropy measurements were conducted with Rev-Fl (10 nM) in a thermocontrolled cuvette (22 °C) within a Perkin Elmer LS50 luminometer fitted with polarizers. Excitation of the peptide was at 490 nm and emission was monitored at 530 nm. Six independent anisotropy measurements were averaged to produce each data point.

Ethidium bromide displacement: A solution of ethidium bromide (1.25 μM) was excited at 546 nm, and its fluorescence emission was monitored at 605 nm before and after the addition of the nucleic acid. For the RRE and TAR constructs, 50 nM of each stand were used; for the polymeric nucleic acids, 0.88 μM or 11 μM base pairs were used. Under these conditions, only a small fraction of the ethidium bromide is bound (less than 20 %). Inhibitors were then titrated into the solution until the fluorescence decrease reached saturation.

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[18] Fluorescent Tat peptides with the sequences fluorescein-CGRKKRRQRRRAHQN-am and fluorescein-GRKKRRQRRRC-am were synthesized and their affinities for TAR31 (K_d = 200 – 400 nM) were too low to be useful for fluorescence anisotropy displacement assays. Other groups have also reported a higher affinity of TAR for Rev than for Tat[17].
[19] These differences cannot be explained by the higher affinity of Rev-Fl for RRE66 than for TAR31, as this should make it more difficult to displace Rev-Fl from the RRE. Furthermore, upon direct titration of the RRE or TAR, changes in the UV-absorbance spectra of **2** and **3** confirm a fourfold preferential binding to the RRE over the TAR (data not shown).
[20] In our initial report[12], **2** and **3** were reported to have the same peptide displacement activities. Additional analysis suggests (albeit within the errors of the measurement itself) a slightly higher activity for **2**.

[21] A crystal structure of rac-**2**/**3** shows the root mean square (RMS) deviation from planarity of the eilatin unit (including hydrogen atoms) is 0.130 Å. Without hydrogen atoms, the RMS deviation from planarity is 0.098 Å (see Ref. [32]). The deviation from planarity of the eilatin itself (without H atoms) is 0.044 Å (see Ref. [1]).

[22] The estimated twist angle is based upon the crystal structure of an analogous unfused system $[Ru(bpy)_2bisisoquinoline]^{2+}$. For details, see: M. T. Ashby, G. N. Govindan, A. K. Grafton, *J. Am. Chem. Soc*. **1994**, *116*, 4801 – 4809.

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[27] In order to calculate binding constants from displacement experiments, the IC_{50} value must be weighted by both the affinity and stoichiometry of each nucleic acid with respect to ethidium bromide as well as the number of ethidium molecules displaced per equivalent of metal complex.

[28] The different values measured by the two assays likely reflect different binding sites and stoichiometries of Rev-FI compared to ethidium bromide.

[29] See the Supporting Information for details.

[30] Previous experiments with the HH-16 hammerhead ribozyme found that Δ-$[Ru(bpy)_2eilatin]^{+2}$ inhibits RNA-dependent RNA phosphodiester bond cleavage independent of the ionic strength of the buffer, which suggests that **2** and **3** bind to nucleic acids predominantly through hydrophobic interactions.[7i] This, taken with the planarity requirement for eilatin, suggests that **2** and **3** bind to nucleic acids by intercalation or some other stacking interaction.

[31] For chiral discrimination between the enantiomers of $[Ru(phen)_3]^{2+}$ by tRNA, see Ref. [7g]. For the chiral discrimination of $[Rh(phen)_2phi]^{3+}$ by bovine immunodeficiency virus TAR see Ref. [7h].

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