Neomycin−Acridine Conjugate: A Potent Inhibitor of Rev-RRE Binding

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The successful replication of many retroviruses requires an ordered pattern of viral gene expression.[1] Small organic molecules that target viral RNA sites and prevent the formation of key RNA−protein complexes are promising candidates for drug discovery.[2,3] Regulatory proteins that act posttranscriptionally are particularly attractive since the RNA sequences they recognize are likely to also serve as coding sequences for other essential viral proteins.[4] The HIV-1 Rev-Response-Element (RRE) serves as the Rev-binding site responsible for the active export of unspliced HIV genomic RNA from the nucleus as well as part of the envelope-protein open reading frame.[5] Because of this dual function, the evolution of resistant variants may be prevented or impeded. Neomycin B and other aminoglycoside antibiotics have been shown to competitively block the binding of the Rev protein to its RNA recognition element (RRE), thus providing an important precedent for the utilization of small molecules to target viral RNA sites.[6,7] The natural antibiotics, however, are rather promiscuous RNA binders and bind the RRE with relatively low affinity.[3a] We hypothesize that by combining structural elements essential for RNA binding with structure-specific determinants, new small molecules with high affinity to the RRE site can be identified.

The high-affinity Rev binding domain within the RRE consists of a stem−bulge−stem structure (Figure 1).[8] The arginine-rich fragment, Rev_{34-50}, binds the RRE with a dissociation constant comparable to that of the full-length Rev protein (Figure 1).[9] The NMR structure of a Rev-RRE complex reveals that binding of the Rev peptide to the RRE initiates G48-G71 and G47-A73 base pairing and forces U72 to bulge out.[10] Since single base bulges are potential high-affinity sites for intercalating agents,[11] we hypothesized that the combination of strong ionic interactions with intercalation ability may lead to potent inhibitors of Rev−RRE binding. A neomycin−acridine conjugate (**2**) has, therefore, been synthesized by covalently linking neomycin B (**1**) to 9-amino-acridine via a short spacer (Scheme 1).[12] We report that neo-acridine (**2**) is a potent inhibitor of Rev−RRE binding. Its affinity to the RRE is two orders of magnitude higher than that of the parent neomycin B (**1**) and approaches that of the Rev peptide.

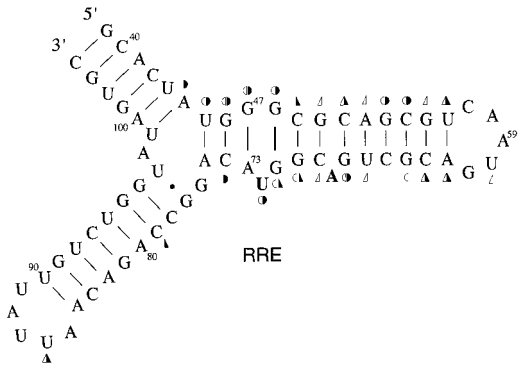
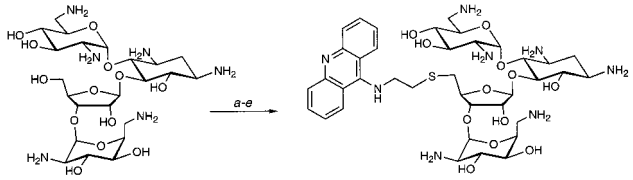


Figure 1. The proposed secondary structure of the 67-nt RRE within domain II of the HIV-1 RNA and the arginine-rich RNA-binding domain of the Rev protein (Rev_{34-50}). The RRE sequence is shown with a schematic summary of the enzymatic protection experiments (see text). Footprinting in the presence of Rev (open half-circle) and neo-acridine (filled half-circle) as well as enhanced cleavage in the presence of Rev (open triangle) and neo-acridine (filled triangle) are shown.

Scheme 1. Synthesis[a] and Structure of Neo-acridine (**2**)[12]



[a]Reagents and conditions: (a) $(Boc)_2O$, DMF, H_2O, Et_3N, 60 °C, 2 h, 72%; (b) 2,4,6-triisopropylbenzenesulfonyl chloride, pyridine, room temperature, 20 h, 75%; (c) $H_2NCH_2CH_2SH$, NaOEt/EtOH, room temperature, 4.5 h, 80%; (d) 9-phenoxyacridine, phenol, 60−75 °C, 1 h, 84%; (e) 4 M HCl/dioxane, $HSCH_2CH_2SH$, room temperature, 5 min, 80%.

Gel-shift mobility assays have been employed to qualitatively study the interactions of Rev_{34-50} and neo-acridine (**2**) with the RRE.[12,13] As illustrated in Figure 2, neo-acridine (**2**) displaces Rev from the Rev−RRE complex much more effectively than neomycin B (**1**). Inhibition curves yield IC_{50} values of 5.9 ± 1.9 and 0.65 ± 0.1 μM for neomycin B and neo-acridine, respectively. While not observed for neomycin B, a binary neo-acridine−RRE complex is formed at ca. 2.5 μM **2**. At this concentration the Rev−RRE complex formation is completely inhibited. This suggests the presence of a single high affinity site that is responsible for disrupting the RNA−peptide complex.[14] Gel shift experiments demonstrate that the relative affinity of neo-acridine (**2**) to the RRE is 2-fold lower than that of the Rev peptide.[12]

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(12) See Supporting Information for experimental details.
(13) Modified Rev_{34-50} peptides of the sequence $_{suc}$TRQARRNRRRRWRERQRAAAAC$_{am}$ have been utilized for all experiments. Succinylation of the *N*-terminus and addition of four alanine residues to the *C*-terminus have been shown by Frankel to enhance the α-helicity of the peptide and, consequently, increase its affinity and specificity to the RRE (see: Tan, R.; Chen, L.; Buettner, J. A.; Hudson, D.; Frankel, A. D. *Cell* **1993**, *73*, 1031−1040). The cys residue at the *C*-terminus is used for fluorescent tagging. In the unlabeled peptide, this residue is blocked as an acetamide SCH_2CONH_2 derivative.

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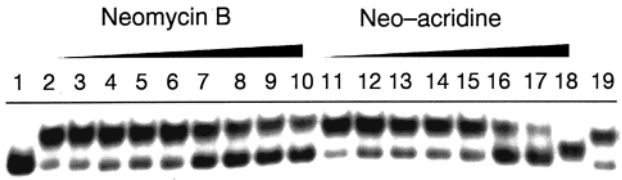

Figure 2. Gel mobility shift of the Rev-RRE complex in the presence of increasing concentrations of neomycin B (**1**) or neo-acridine (**2**).[12] All lanes contained 25 nM RRE with trace 5′-^{32}P labeled RRE and lanes 2−19 contain 2 μM Rev. Lane 1, RRE alone; lanes 2 and 19, RRE and Rev; lanes 3−10, 0.5 μM, 1 μM, 1.25 μM, 2.5 μM, 3.75 μM, 5 μM, 7.5 μM, and 10 μM neomycin B, respectively; lanes 11−18, 25 nM, 50 nM, 100 nM, 200 nM, 500 nM, 750 nM, 1.25 μM, and 2.5 μM neo-acridine, respectively.

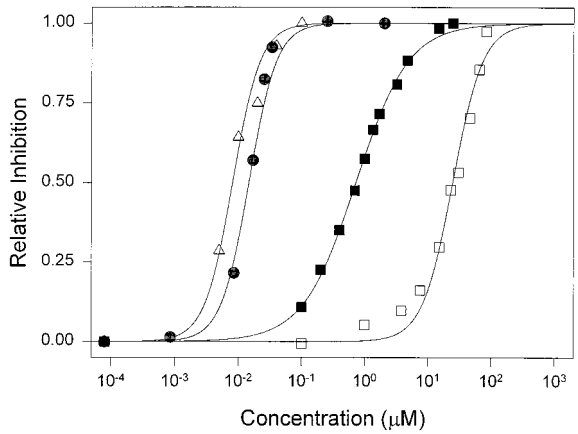

Figure 3. Inhibition of Rev-Fl binding to the RRE by Rev (△), neo-acridine (●), neomycin B (■), and 9-aminoacridine (□) as determined by fluorescence anisotropy displacement measurements.[12]

Fluorescence anisotropy has been employed to quantitatively determine the RRE binding affinity of Rev−RRE inhibitors.[12] Inhibition is measured by the displacement of a fluorescein-labeled Rev_{34-50} peptide (Rev-Fl) off the RRE construct (Figure 1).[12,13] Competition experiments yield IC_{50} values of 24 ± 1 μM for 9-aminoacridine, 0.8 ± 0.2 μM for neomycin B, 15 ± 1 nM for neo-acridine (**2**), and 12 ± 5 nM for the Rev peptide (Figure 3). Since gel-shift assays suggest a single high-affinity site for both high-affinity ligands, K_i values of 1.0 ± 0.8 and 1.5 ± 0.3 nM can be calculated for the Rev peptide and neo-acridine (**2**), respectively.[12] In agreement with the gel-shift data, the affinity of neo-acridine (**2**) to the RRE is only 2-fold lower than that of the Rev peptide.

To locate the binding sites of Rev_{34-50} and neo-acridine (**2**) on the RRE, enzymatic protection experiments with three ribonucleases, RNase T1, RNase A, and RNase V1, have been conducted.[12] Footprinting for both Rev and neo-acridine (**2**) is located around the G46-G48 bulge region (Figure 1). At low concentrations (0.5−2 μM), both the Rev peptide and neo-acridine (**2**) show protection at nucleotides U45, G46, G47, G48, G67, and U72. Neo-acridine (**2**) and Rev are, therefore, competing for the same site on the RRE. Intriguing differences between the cleavage patterns observed for neo-acridine (**2**) and the Rev peptide are localized around the bulged-out U72 (Figure 1). In the presence of the Rev peptide, G70 and G71 are protected and enhanced cleavage is observed at C69. Protection by neo-acridine (**2**) at nucleotides A44 and C74 is not observed with the Rev peptide. Importantly, enhanced cleavage appears at nucleotides C49, G70, and G71 in the presence of neo-acridine. These data suggest that the acridine moiety of neo-acridine (**2**) interacts near or at the bulged U72 while the neomycin B portion flanks the nucleotides A44-G47.[14] The enhanced RNase T1 cleavage observed at G71 in the presence of neo-acridine contrasts the protection observed for the Rev peptide at the same site. The increased single-stranded nature of the G71 suggests that neo-acridine may disrupt the G48-G71 base pair.[15] These results, together with gel-shift and fluorescence anisotropy data, suggest neo-acridine competitively inhibits Rev−RRE complex formation by binding at the same region of RRE as the Rev peptide.[16]

To the best of our knowledge, neo-acridine (**2**) is the strongest competitive inhibitor of Rev−RRE binding. It binds the RRE with an inhibition constant of 1.5 nM and effectively disrupts the Rev−RRE complex. The affinity of neo-acridine to the RRE is only 2-fold lower than that of the Rev peptide. Our results demonstrate that (a) small molecules can effectively interfere with protein−RNA interactions, (b) synthetic ligands can achieve very high RNA affinity, approaching that of the natural RNA-binding domains on proteins, and (c) the combination of different binding modes (e.g., ionic and intercalation) is a powerful approach for enhancing the RNA affinity of synthetic binders. Small molecules that target viral RNA sites and prevent the formation of pivotal regulatory RNA−protein complexes are promising candidates for antiretroviral drug discovery.

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Supporting Information Available: Synthetic procedures and analytical data for all new derivatives as well as procedures and data for gel-shift assays, fluorescence anisotropy measurements, calculations of K_d and K_i values, and enzymatic protection reactions (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(14) A second lower affinity RRE binding site is filled at higher neo-acridine concentrations (5−10 μM). Footprinting data at these concentrations suggest a lower affinity neo-acridine binding site located around A68, another bulged nucleotide. See ref 12.

(15) A possible explanation is the formation of a G48·U72 base pair and expulsion of G71 into solution upon intercalation of the acridine moiety.

(16) The Rev peptide has been shown to have the same RNA binding domain as the full length Rev protein. See ref 9 and: Daly, T. J.; Rennert, P.; Lynch, P.; Barry, J. K.; Dundas, M.; Rusche, J. R.; Doten, R. C.; Auer, M.; Farrington, G. K. *Biochemistry* **1993**, *32*, 8945−8954.