Structure–activity relationships of aminoglycoside-arginine conjugates that bind HIV-1 RNAs as determined by fluorescence and NMR spectroscopy

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Received 5 August 2004; revised 11 October 2004; accepted 14 October 2004

Available online 27 October 2004

Edited by Christian Griesinger

Abstract **We present here a new set of aminoglycoside-arginine conjugates (AACs) that are either site-specific or per-arginine conjugates of paromomycin, neamine, and neomycin B as well as their structure–activity relationships. Their binding constants (K_D) for TAR and RRE RNAs, measured by fluorescence anisotropy, revealed dependence on the number and location of arginines in the different aminoglycoside conjugates. The binding affinity of the per-arginine aminoglycosides to TAR is higher than to RRE, and hexa-arginine neomycin B is the most potent binder (K_D = 5 and 23 nM, respectively). The 2D TOCSY NMR spectrum of the TAR monoarginine-neomycin complex reveals binding at the bulge region of TAR.**
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Keywords: Aminoglycoside-arginine conjugate; HIV-1 TAR, RRE RNA; Fluorescence anisotropy; 2D-TOCSY NMR TAR-NeoR1

1. Introduction

The complexity and diversity of RNA structures offer attractive targets for small molecule ligands to be used as pharmacological agents [1]. Aminoglycosides have found clinical use as antibacterial agents, due to their ability to specifically bind bacterial ribosomes [2]. Aminoglycosides also interact with a large number of other RNAs including the two essential elements of the HIV genome, Rev responsive element (RRE) and the transactivation responsive element (TAR) [3]. For example, the binding of neomycin B to HIV TAR in the minor groove leads to conformational changes in TAR, thus restricts HIV-1 transactivator protein, Tat, binding at the major TAR-RNA groove [4,5]. The molecular basis of Rev-RRE recognition requires interaction of the 17-mer arginine-rich Rev (residue 33–50) peptide with a bulge portion of RRE IIB RNA construct, which forms an A-form RNA duplex with a 3×2 internal loop [6]. Non-canonical pairs G●A and G●G are essential for Rev recognition [7] (Fig. 1). The Tat–TAR interaction involves an arginine-rich Tat peptide (residues 49–57) and the three-nucleotide UCU bulge region of TAR, flanked by two double stranded stems (e.g., [8,9]) (Fig. 1). Peptides carrying an arginine-rich sequence of Tat and even a nona-arginine peptide bind TAR RNA at the UCU bulge with high affinity and specificity (e.g., [10,11]). Importantly, it was reported that not only Tat peptide binds TAR RNA with high affinity, but the Rev peptide does as well, functionally substituting for Tat [11,12]. Thus, Rev peptide labeled with carboxymethyl rhodamine (RhdRev; Fig. 1) was used in this study as a fluorescent probe for both TAR and RRE IIB RNA constructs.

Arginine- and lysine-rich basic peptides comprise a common motif of RNA recognition by proteins. For example, HIV-1 Tat and Rev proteins mediate their interactions with the viral RNAs via arginine-rich motif [10]. Although the dominant contributions of the arginine side-chains may differ between complexes, the ability of the guanidinium groups of the arginine side chains to be involved in electrostatic interactions, hydrogen bond formation and stacking interactions makes arginine an important moiety for RNA recognition [13]. Attempts to mimic the arginine-rich peptides led to the development of novel RNA ligands, which utilize a diverse set of building blocks [14]. Arginine-rich RNA-binding peptides and peptidomimetics have provided a good scaffold for RNA-targeting drug design, since they are short, conformationally diverse and contact RNA with high affinities and specificities.

We have recently shown that arginine-aminoglycoside conjugates (AACs) are far more efficient anti-HIV-1 agents than aminoglycosides, as these molecules comprise the RNA binding ability of aminoglycosides and the specific binding of arginine moiety to HIV-1 TAR RNA. AACs were designed to bind HIV TAR RNA and to inhibit trans-activation by Tat protein [14–16]. AACs are antagonists of the HIV-1 Tat protein basic domain and structurally are peptidomimetic compounds with different aminoglycoside cores and different numbers of arginines [16,17]. Along with inhibition of Tat transactivation step in HIV life cycle, AACs exert a number of other activities, closely related to Tat antagonism. For example, hexa-arginine neomycin B conjugate (NeoR6) (Fig. 2) inhibits the following functions of extracellular Tat protein: upregulation of the HIV-1 viral entry via CXC chemokine

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doi:10.1016/j.febslet.2004.10.038

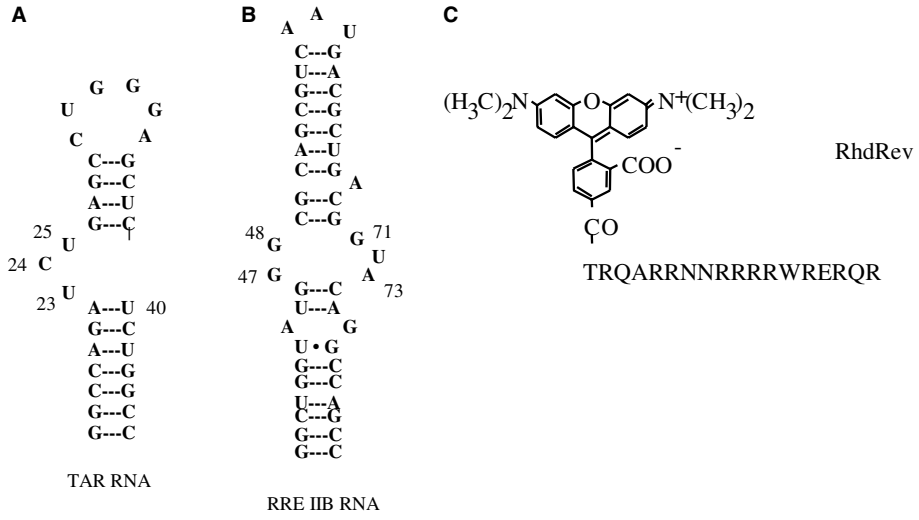
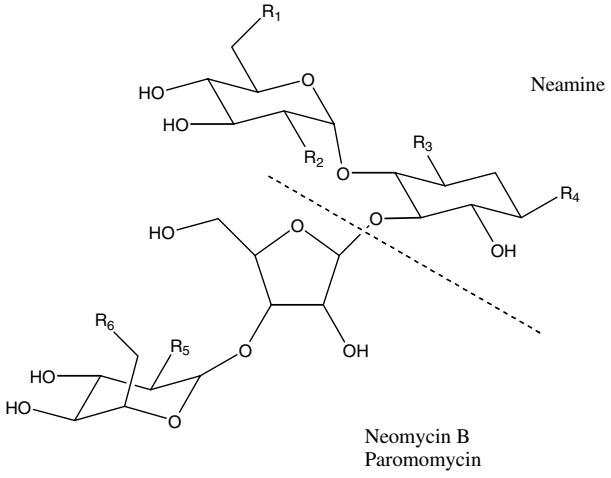


Fig. 1. Structures of (A) TAR and (B) RRE IIB RNA oligonucleotides and (C) RhdRev Fluorescent probe.

receptor type 4 (CXCR4), increase of viral production, suppression of CD3-induced proliferation of lymphocytes, and upregulation of CD8 receptor [17]. We have shown that NeoR6 and tri-arginine gentamycin conjugate (R3G) inhibit binding of HIV-1 particles to cells, probably by blocking the CXCR4 co-receptor [17,18]. This was substantiated by the finding that NeoR6 competes with the binding of the monoclonal antibody 12G5 to CXCR4; it also competes with the binding of CXCR4-stromal cell derived factor 1α (SDF-1α) to CXCR4 [17,19,20]. Moreover, NeoR6 crosses the blood brain barrier when administered systemically and enters into various brain tissues [21]. All of the above data suggest that AACs may lead to an extremely important class of anti-HIV drugs. An additional interesting property of AACs (e.g., NeoR6 and R3G) is inhibition of bacterial (and to a lesser extent, mammalian) RNAse P activity ~500 fold more efficiently than neomycin B [22]. We have recently shown the capacity of several AACs to inhibit peptidyl transferase activity [23].



	R_1	R_2	R_3	R_4	R_5	R_6
Neomycin	NH2	NH_2	NH_2	NH_2	NH_2	NH_2
NeoR6	Arg	Arg	Arg	Arg	Arg	Arg
NeoR1	Arg	NH_2	NH_2	NH_2	NH_2	NH_2
NeoR1	NH_2	NH_2	NH_2	NH_2	NH_2	Arg
NeoR2	Arg	NH_2	NH_2	NH_2	NH_2	Arg
Neamine	NH_2	NH_2	NH_2	NH_2	--	--
NeamR1	Arg	NH_2	NH_2	NH_2	--	--
NeamR4	Arg	Arg	Arg	Arg	--	--
Paromomycin	OH	NH_2	NH_2	NH_2	NH_2	NH_2
ParomR1	OH	NH2	NH_2	NH_2	NH_2	Arg
ParomR5	OH	Arg	Arg	Arg	Arg	Arg

Fig. 2. Schematic representation of aminoglycoside-arginine conjugates and amino-glycosides used in this study. All AACs were prepared as acetate salts. Hexa-arginine neomycin conjugate (NeoR6); a 1:1 mixture of two mono-arginine neomycin conjugates (NeoR1); a di-arginine neomycin conjugate of neomycin B (NeoR2); a mono-arginine neamine conjugate (NeamR1); a tetra-arginine neamine conjugate (NeamR4); a mono-arginine paromomycin conjugate (ParomR1); and a penta-arginine paromomycin conjugate (ParomR5).

The structure–function relationship of AACs with respect of RNA binding is an important issue for drug development. We hypothesized that similar to the recognition of 16S RNA by rings I and II of neomycin B class of antibiotics, e.g., neamine and paromomycin [24] (Fig. 2), being conjugated to arginine would be sufficient to mediate specific interactions with the HIV-1 RNAs. Thus, syntheses and characterization of site-specific mono-arginine conjugates of neamine, paromomycin and neomycin B, as well as tetra-arginine neamine, penta-arginine paromomycin, di- and hexa-arginine neomycin B conjugates (Fig. 2) were undertaken. Their binding to HIV-1 TAR and RRE RNA, as presented by fluorescence anisotropy, points that hexa-arginine neomycin B (NeoR6) exerts the highest binding affinity to both RNAs. Although the mono-arginine aminoglycoside conjugates bind TAR and RRE with significantly lower affinity than the per-arginine conjugates, the site of binding to TAR, as presented in this paper by 2D ^{1}H NMR, reveals binding at the bulge region of TAR.

2. Materials and methods

2.1. Materials

The procedure for the synthesis and purification of NeoR6 [17], and for NeoR1, NeoR2, NeamR1, NeamR4, ParomR1 and ParomR5 was reported [25,26].

2.2. Fluorescence anisotropy measurements

A 31 residue TAR RNA fragment containing residues 19–44 HIV-1 LTR, the high affinity Tat binding site, and a 41 residue RRE IIB RNA fragment containing the high affinity Rev binding site (Fig. 1) were purchased from Dharmacon Research Inc (Boulder, CO); they were deprotected using the company's buffer and protocol. Rhodamine-Rev peptide (residues 34–50 TRQARRNRRRRWRERQR) (RhdRev) was prepared as previously described [27] and used as a fluorescence probe for binding to the 31-mer TAR RNA and to the 41-mer RRE RNA. Tar and RRE RNA were annealed by heating to 95 °C with gradual cooling to room temperature. All stock solutions were prepared in nuclease-free water and were diluted with appropriate buffers prior to use. RNA concentrations were determined spectrophotometrically at 260 mm and the samples were re-annealed each time.

Binding of RhdRev to HIV TAR RNA and AACs competition with RhdRev on binding to TAR and RRE IIB RNAs were examined by fluorescence anisotropy [27,28], preformed on SLM-Aminco model 8100 Series 2 spectrometer (Spectronic Instruments) equipped with a thermostat accurate to ±0.1 °C. The TAR binding samples (0–200 nM) were excited at 550 nm and monitored at 580 nm; the integration time was 4 s. Every point consists of 10–20 measurements and their average values were used for calculation. Measurements were performed at 20 °C in a buffer solution containing 85 mM NaC1, 2 mM KC1, 0.5 mM $MgC1_2$, 0.5 mM $CaC1_2$ and 10 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] (pH 7.5) [27]. The RhdRev tracer concentration (0–70 nM) was determined spectroscopically at 550 nm using a molar excitation coefficient of $6.00 \times 10^4\ M^{-1}\,cm^{-1}$ [29]. Different AAC concentrations (0–500 nM in 20 nM increments) were used for the competition studies.

2.3. Determination of binding constants

Eq. (1) was used for the calculation of the dissociation constants (K_d) of the interaction of RNA and the fluorescent tracer RhdRev [29,28]

$$A = A_0 + \Delta A\{[\mathrm{RNA}]_0 + [\mathrm{tracer}]_0 + K_d - [([\mathrm{RNA}]_0 + [\mathrm{tracer}]_0 + K_d)^2 - 4[\mathrm{RNA}]_0[\mathrm{tracer}]_0]^{1/2}\}/2, \quad (1)$$

where A and A_0 are the fluorescence anisotropy of RhdRev (tracer) in the presence and absence of RNA, respectively, and ΔA is the difference between the fluorescence anisotropy of the tracer at extrapolation to infinite concentration of RNA minus the fluorescence anisotropy in the absence of RNA. $[RNA]_0$ and $[tracer]_0$ are the initial concentrations of RNA and the fluorescent tracer (RhdRev), respectively.

Eq. (2) [29] is used for the determination of K_D values in the competition-binding assay

$$[\mathrm{AAC}]_0 = [K_D(A_\infty - A)/K_d(A - A_0) + 1][[\mathrm{RNA}]_0 - K_d(A - A_0)/(A_\infty - A) - [\mathrm{tracer}]_0(A - A_0)/(A_\infty - A)], \quad (2)$$

where K_D is the dissociation constant of RNA–AAC complex; $[AAC]_0$ is the initial concentration of the aminoglycoside-arginine conjugates. Both K_d and K_D were determined by non-linear curve fitting of the experimental points using Kaleidagraph and Eqs. (1) or (2) described above and presented as mean values of three independent measurements.

2.4. 2D-TOCSY NMR of TAR in the presence and absence of NeoR1

All NMR experiments were performed on Varian Inova 600 MHz spectrometers using samples of 31-nucleotide TAR RNA at 0.8 mM, in 10 mM sodium phosphate buffer (pH 6.5), and 20 mM NaCl. Homonuclear TOCSY spectra in D_2O were recorded at 35 °C. Spectra were apodized via cosine-bell window functions in each dimension and zero-filled once. The spectra were processed with NMRpipe [30] and analyzed with SPARKY [31]. NOESY spectra were acquired from 10 to 35 °C.

3. Results and discussion

3.1. Rationale

Our recent studies [17] suggest that NeoR6 targets the Tat transactivation step in the HIV-1 life cycle. NeoR6 displays high in vitro inhibition of Tat–TAR interaction in a concentration range of Tat-R52 peptide [16,17]. A model structure for the TAR-NeoR6 complex, which complied with available biochemical data, suggested possible binding of NeoR6 to TAR RNA at the bulge region [17], the site for Tat basic peptide binding [12,13]. To this end, the mode of binding of different aminoglycoside site-specific arginine conjugates and the function(s) of the other arginine moieties on TAR or RRE RNA binding were not yet investigated. Towards this goal, a set of different aminoglycosides conjugated to 1–6 arginines was prepared, and their binding to TAR and RRE was measured as well as the binding of site specific mono-arginine neomycin conjugate(NeoR1) to TAR was examined by 2D TOCSY ^1H NMR.

3.2. Binding of aminoglycoside-arginine conjugates to TAR and RRE IIB RNA constructs

The initial experiment aimed at determining whether RhdRev peptide binding to TAR is similar or not to its interaction with RRE IIB. TAR and RRE IIB constructs (Fig. 1) were used in this study. The fluorescence probe (10 nM) was titrated with increasing concentrations of RRE IIB or TAR (0–60 nM) in a buffer solution containing 85 mM NaCl. The fluorescence anisotropy changes were plotted as a function of increasing concentration of RRE IIB or TAR. Non-linear curve fitting was used to determine the K_d of RhdRev-RNA complexes. The isotherms for RhdRev binding to RRE IIB and TAR (Fig. 3A

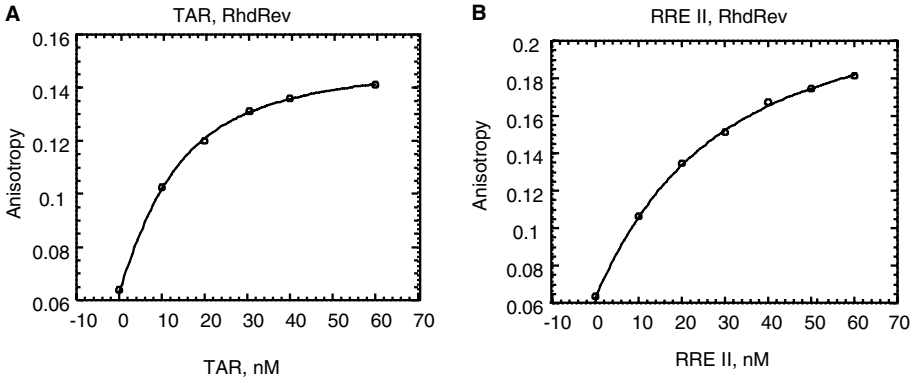


Fig. 3. Fluorescence anisotropy of the fluorescently labeled Rev ($RhdRev_{34–50}$ 10 nM) as a function of (A) TAR RNA concentration, and as a function of (B) RRE IIB RNA concentration.

Table 1
Dissociation constants (K_d, K_D, nM) of AACs binding to TAR and RRE RNA and IC_{50}, determined by competitive experiments with RhdRev[A]

Compound	K_D TAR/RhdRev[a]	IC_{50} TAR/RhdRev[b]	K_D RRE IIB[c]	IC_{50} RRE IIB[d]
RhdRev[e]	7.3 ± 0.5	–	16.6 ± 2.7	–
NeoR6	5.0 ± 0.2	19.9 ± 2.7	23.3 ± 0.9	72.7 ± 10.1
NeoR2	>500[f]	>500[f]	111.5 ± 3.7	207.9 ± 21.1
NeoR1	>500[f]	>500[f]	200.2 ± 5.9	352.8 ± 31.9
ParomR5	15.1 ± 0.4	34.3 ± 5.7	95.9 ± 2.2	149.4 ± 12.3
ParomR1	>500[f]	>500[f]	254.2 ± 7.8	406.8 ± 41.0
NeamR4	30.1 ± 1.2	82.8 ± 7.7	54.7 ± 1.1	118.8 ± 13.5
NeamR1	>500[f]	>500[f]	233.4 ± 4.5	342.4 ± 29.5

[a]K_D and [b]IC_{50} inhibitory concentration (IC_{50}) determined by competition of AACs with RhdRev for TAR RNA binding; [c]K_D and [d]IC_{50} determined as competition of AACs with RhdRev for RRE IIB RNA binding; [e]measured as direct binding of RhdRev to TAR and RRE IIB RNA; [f]20 μM of mono and di-arginine aminoglycoside derivatives failed to completely expel RhdRev from the complex with TAR, thus $IC_{50's}$ and K_D's are >500 nM and could not be accurately determined.
The K_d, K_D and IC_{50} values are means of three independent measurements.
[A]The K_D (μM) values 1.18 and 8.3 for neomycin B and paromomycin binding to RRE respectively; and IC_{50} (μM) values 28 and 21 for neamine binding to TAR and RRE respectively were previously published [28,29,39].

and B) and the K_d values for Rhd-Rev-RRE IIB complex (16 nM) and for RhdRev-TAR (7 nM) (Table 1) exhibit high binding affinity of RhdRev to TAR. This is in line with previous finding that Rev peptide binds TAR, functionally substituting for Tat (10–12). Thus, RhdRev was used to determine the K_D of AACs-TAR and AACs-RRE complexes. In these experiments to 10 nM of the fluorescent tracer (RhdRev), fixed amount (20 nM) of TAR or RRE IIB RNA was added (in a buffer containing 85 mM NaCl). The complexes of the tracer and the corresponding RNA were further titrated with various AACs and anisotropy values were recorded (Fig. 4A–C). Using non-linear curve fitting, the dissociation constants (K_D) of AACs-TAR and AACs-RRE IIB RNA complexes were determined (Table 1). The titration of RhdRev-RRE IIB complex with AACs exhibited an expected decrease of anisotropy, indicating release of the fluorescent tracer from the complex, and resulted in accurate K_D values for AACs-RNA complexes. When similar competition experiments were performed with RhdRev-TAR, only AACs with several arginine side chains, such as NeoR6, ParomR5 and NeamR4, efficiently compete with RhdRev for TAR binding (Table 1) (Fig. 4A–C). The AACs, featuring one or two arginine side chains, failed to completely expel the tracer from its complex with TAR and the residual anisotropy did not return to its initial value; thus, their K_D values could not be accurately measured.

Noteworthy, the binding of NeoR6, ParomR5 and NeamR4 to TAR appeared to be more efficient than to RRE IIB (Table 1). Overall, the measurements confirmed that NeoR6 binds with the highest affinity to both RRE IIB (K_D 23 nM) and TAR (K_D 5 nM) (Table 1), comparable to the natural ligands, Rev residues 34–50 (40 nM) and Tat residues 49–57 (12 nM). ParomR5 binds TAR with K_D (15 nM), 3 times weaker than NeoR6 and binds RRE IIB with K_D (96 nM) about 4 times weaker than NeoR6. NeamR4 binds TAR with K_D (30 nM) 6 times weaker than NeoR6, while its binding to RRE IIB, K_D (55 nM) is only 2 times weaker than to NeoR6 (Table 1), i.e., NeamR4 binds RRE IIB more efficiently than ParomR5. AACs with several arginines clearly bind TAR better than RRE IIB. For comparison with published data (e.g., [32]), the IC_{50} values for AACs competition with RhdRev for TAR and RRE IIB were also determined (Table 1).

It is now well established that the dissociation constants of aminoglycoside-RRE IIB RNA complexes are in the micromolar range. Even neomycin B that binds RRE IIB with the highest affinity (K_D of 1.18 μM [29]) is about 550 times weaker than hexa-arginyl neomycin B (NeoR6) and 6–7 times weaker than mono- and di-arginine derivatives (NeoR1 and NeoR2). This indicates that one arginine conjugated to neomycin B already significantly increases its binding affinity to HIV-1 regulatory RNAs. Other aminoglycoside derivatives, e.g., guanylated aminoglycosides (e.g., [32]) or the tetra-γ-guanidinobutyrate kanamycin A derivative (GB4K [15]), bind TAR or RRE RNAs much weaker than AACs. Similarly, their anti-HIV-1 activities are much lower than the respective arginine-aminoglycoside derivatives [16–18]. This might be due to different binding sites on HIV RNAs for peptide and AAC than other aminoglycoside derivatives. For example, we have shown that GB4K binds to a different region of TAR RNA than Tat peptide (R52) or AACs [15,16]. Another example is neomycin B, which binds RRE IIB in the major groove, at the lower stem-bulge region of the construct, close to, but not at the Rev peptide binding site (e.g., [33]). In the case of TAR RNA, neomycin B binds in the minor groove in a region very different from the Tat binding site [4]. Both TAR and RRE bind peptides in the major groove of the corresponding RNA constructs. At least in the case of TAR RNA, tetra- to hexa-arginylated AACs binding pattern is in accordance with the peptide-like binding [10–12,19]. Based on our previous in vitro binding studies to TAR RNA and anti-HIV-1 activities of various arginine and guanidine derivatives of aminoglycosides, we proposed that the α-amino groups of the arginine moieties play an important role in TAR RNA site specific recognition and in anti-HIV-1 activity [15,17]. Thus, AACs bear features of both aminoglycosides and peptides.

We present here the structure-binding affinity of AACs to RNA, the K_D values of AACs binding to TAR and RRE IIB (Table 1) as a function of the number of arginine side chains and the number of rings of the aminoglycoside core (Fig. 2). NeoR6 displays the highest affinity to TAR and RRE IIB (i.e., the lowest K_D values), however, there is not much difference in the affinity of NeamR4 and ParamR5. NeamR4 renders even lower K_D value (higher affinity) for RRE than ParomR5 does. Thus, not only are the number of rings and arginine moieties essential for efficient RNA binding, but also the conjugation of arginine to the methylamino group (R_1) of ring I aminoglycoside is important to improve binding affinity to RNA. While neamine was suggested as a minimal RNA recognition unit [24], arginine conjugated to rings I and II is not sufficient for

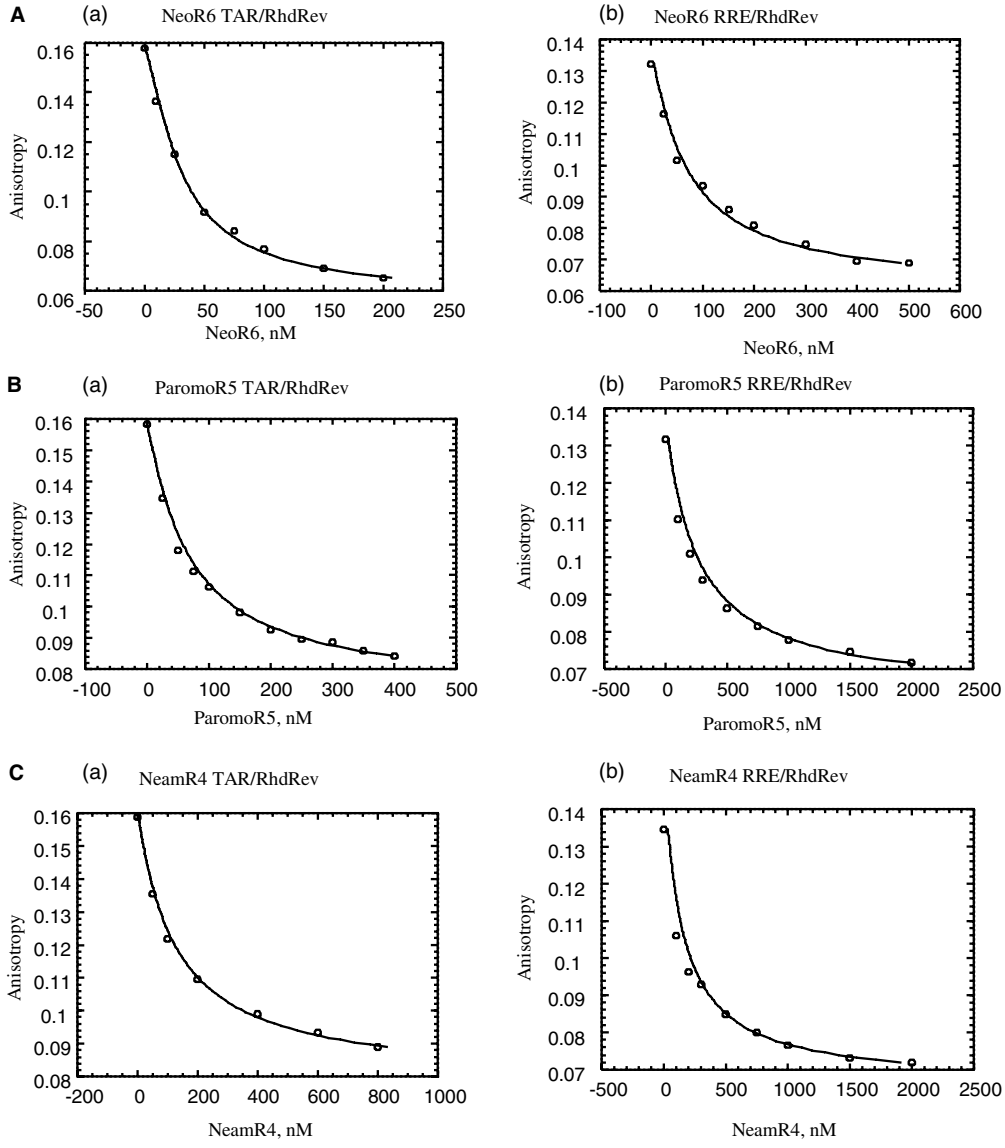


Fig. 4. Fluorescence anisotropy of RhdRev (10 nM) solution containing [A] (a) TAR RNA (20 nM) and (b) RRE IIB RNA (20 nM), as a function of various concentrations of NeoR6; [B] (a) TAR RNA (20 nM) and (b) RRE IIB RNA (20 nM), as a function of various concentrations of ParomR5; [C] (a) TAR RNA (20 nM) and (b) RRE IIB RNA (20 nM), as a function of various concentrations of NeamR4.

effective RNA binding. Thus, NeamR4 is a significantly weaker RNA binder than NeoR6, suggesting that rings III and IV and increased number of arginine moieties improve binding affinity of AACs to RRE and TAR RNAs.

Several structural studies of free and ligated RRE-IIB have been published in the past few years (e.g., [33,34]). It was found that the stem-loop RRE-IIB changes conformation upon binding of Rev. Based on the available structures and the fact that NeoR6 competes with Rev peptide (presented in this study), assuming the same binding site or induced conformational changes in the RRE-IIB stem-loop region similar to that of Rev, we anticipate that the interactions of arginine moieties of NeoR6 are likely to imitate at least in part the interactions of the arginine side chains of Rev with RRE. This should be further investigated. Our experimental results suggest that the arginine moieties at position R_1 on ring I, and R_3 and R_4 on ring II (Fig. 2) are important for binding. The important contribution of arginine at position R_1 is demonstrated by the lower affinity of ParomR5 to RRE IIB and TAR compared to NeamR4. Thus, even though ParomR5 consists of 4 rings and 5 arginine moieties it lacks arginine at position R_1 (Table 1 and Fig. 2), which is probably the reason for the lower affinity. The most potent binder, NeoR6, forms additional arginine-RRE interactions compared to NeamR4. These observations are also in accord with the differences in arginine side chain dynamics of Rev-RRE complex [34].

3.3. TAR-NeoR1 complex binding site as determined by ^1H NMR

While in vitro assays confirm inhibitions, they do not explicitly show that the ligand binds to the designated site and they do not preclude its binding to other sites. However, NMR not only shows whether a ligand binds to the specific target but also can reveal all sites on the target that interact with the ligand [35]. This requires resonance assignments for the target

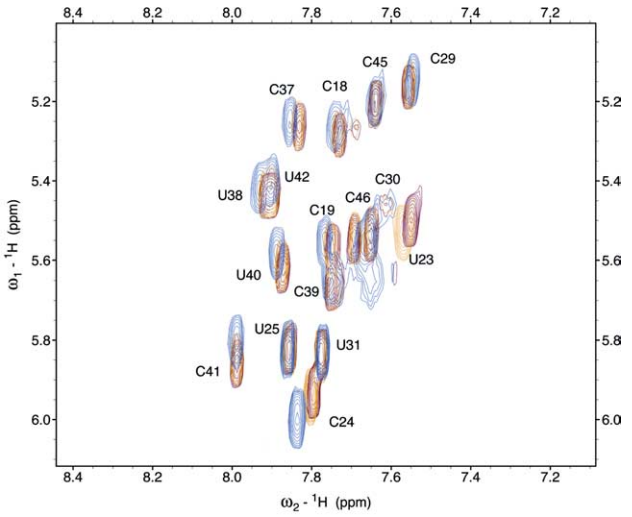


Fig. 5. Chemical shift changes of TAR RNA upon NeoR1 binding. ^{1}H NMR TOCSY spectra of TAR RNA (0.8 mM) in the absence (red) and presence of either 0.3 mM (yellow) or 0.9 mM NeoR1 (blue). The spectra were acquired at 35 °C.

TAR RNA, which are available to us [35,36]. NeoR1 was selected as a simpler form for the NMR study. As anticipated, the NMR data demonstrate binding to the 5′ bulge of TAR, i.e., to the site of Tat protein binding. The 5′ bulge contains three pyrimidine residues (U23, C24 and U25) that can be monitored by their H5–H6 cross-peaks in 2D TOCSY spectra (Fig. 5). These can be compared with the H5–H6 cross-peaks due to other pyrimidine residues. The overlaid TOCSY spectra with increasing amounts of NeoR1 added to TAR show the largest chemical shift changes for U23 and C24, with smaller changes for residues up to three base-pairs away from the bulge. None of the signals from loop residues were altered upon binding NeoR1. Experiments with aminoglycoside conjugates containing multiple arginines also showed selective perturbations in the pyrimidine resonances of bulge residues; however, the spectra for NeoR1 are shown, since the NeoR1 complex was used for further NOE experiments, because all resonances of the ligand are readily assigned. Unfortunately, the NeoR1 complex exhibits a lack of intermolecular NOE cross-peaks in any NOESY spectrum acquired from 10 to 35 °C (not shown). The NOE spectra revealed shifts in RNA peaks but no useful additional information is obtained, since the shifts occurred in the bulge region. The TOCSY spectra of TAR–NeoR1 complex are in line with our published footprinting analysis of the TAR-AACs (R3G and R4K) and TAR–Tat R52 complexes [16]. While binding to the bulge was also seen with promazines as ligands [37], the pattern of shifts is significantly different [37] suggesting that NeoR1 binds in a different fashion than promazines to TAR although it binds in the same vicinity.

4. Conclusions

The development of a new class of HIV-1 inhibitors, targeting viral components other than HIV reverse transcriptase or protease, is of special importance in view of the failure, in many cases, of the current antiretroviral therapies, and due to the emergence of highly variable resistant HIV-1 strains. A current direction in this field is the development of HIV Tat and Rev inhibitors, which may be critical for anti-AIDS strategies.

We present here a new set of AACs, which are either site-specific or per-arginine conjugates of aminoglycosides: neomycin B, paromomycin and neamine, and their binding affinities to TAR and RRE IIB. The hexa-arginine neomycin B conjugate reveals the highest binding affinity either to TAR or RRE IIB. AACs with several arginine groups bind TAR better than RRE IIB. Despite the low efficient binding of monosubstituted AACs, the 2D TOCSY NMR spectra of the TAR-NeoR1 complex reveal binding at the bulge region of TAR. The role of a site-specific arginine and the function(s) of the other arginine moieties of per-arginine conjugates of different aminoglycosides are being investigated. The new AACs exert a number of activities related to Tat antagonism [18,38]. AACs not only target two essential structural elements of the HIV genome but also represent a novel family of inhibitors of viral entry into human cells. It is worth mentioning that the new AACs are not toxic to a large variety of human cell cultures measured up to 500 μM [18], nor to mice (e.g., NeoR6) given two single intravenous doses of 25 mg/kg of body weight over the course of 2 h.

Acknowledgements: This work was supported by internal grants of the Weizmann Institute of Science to Aviva Lapidot and by NIH grant AI46967 to T.L. James.

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