Arginine-aminoglycoside conjugates that bind to HIV transactivation responsive element RNA in vitro

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Received 17 November 1998; received in revised form 7 January 1999

Abstract HIV gene expression is crucially dependent on binding of the viral Tat protein to the transactivation RNA response element. A number of synthetic Tat-transactivation responsive element interaction inhibitors of peptide/peptoid nature were described as potential antiviral drug prototypes. We present a new class of peptidomimetic inhibitors, conjugates of L-arginine with aminoglycosides. Using a gel-shift assay and affinity chromatography on an L-arginine column we found that these compounds bind specifically to the transactivation responsive element RNA in vitro with K_d values in the range of 20–400 nM, which is comparable to the K_d of native Tat bound to the transactivation responsive element (10–12 nM). Confocal microscopy studies demonstrated that fluorescein-labelled conjugate penetrates into live cells. High affinity to the transactivation responsive element, low toxicity, and relative simplicity of synthesis make these compounds attractive candidates for antiviral drug design.

Key words: Aminoglycoside-arginine conjugate; Transactivation responsive element RNA binder; HIV Tat/transactivation responsive element inhibition; Drug design

1. Introduction

Human immunodeficiency virus (HIV) gene expression depends on the interaction of the viral regulatory protein, Tat, with the transactivation responsive element (TAR), a stem-loop RNA structure found at the 5′ end of all HIV-1 transcripts [1]. TAR is a positive enhancer that stimulates the synthesis of productive transcripts and is unique in terms of eukaryotic transcription control because it is only functional as an RNA element. Activation by Tat is entirely dependent on the presence of the TAR RNA sequence and it is known that Tat activates expression by specifically binding to TAR, which increases viral mRNA production several hundred-fold by stimulating the elongation capacity of RNA polymerase II [2]. The Tat interaction with TAR is mediated by a nine amino acid region RKKRRQRRR (residues 49–57) of the protein [3,4]. Both the Tat protein and the specific binding peptide form tight complexes with the TAR RNA in vitro. Studies with peptides suggest that a single properly positioned arginine in a strand of lysines provides the only sequence specific contact with the three nucleotide bulge on the TAR RNA [4–7] by forming a network of hydrogen bonds, called by Frankel et al. [8] an 'arginine fork'. In particular, a synthetic peptide R52 (YKKKRKKKKKA), containing only one arginine residue at position 52, binds to TAR with the same affinity as wild-type Tat 49–57 [4], which contains five arginine residues.

NMR structural studies of the Tat binding sites of HIV and bovine immuno deficiency virus (BIV) TAR demonstrate that the conformational changes in the arginine binding site can be induced by a number of ligands carrying a guanidinium group [9], such as a peptide that mimics the basic region of the Tat protein [10–12], a single arginine [13,14] or L-argininamide [15]. NMR structures show that ligands are bound in the major groove of the RNA [9–15]. The RNA bulge structure allows ligands to access the major groove of TAR [10–12,16], which induces folding in the bulge and formation of unusual base-triples [11–15]. Based on NMR studies, the HIV Tat peptide/TAR complex can be modeled with the basic α-helix lying in the major groove of the RNA, such, that important interactions of a putative specificity-endowing arginine are maintained and a slight widening of the major groove is entailed [17,18]. In contrast, the 17-mer BIV Tat recognition domain folds into a β-hairpin and penetrates in an edge-on orientation deep into a widened major groove of the 28-mer of the BIV TAR RNA [11].

Since the structural basis of the HIV Tat-TAR interaction was postulated [9,10,15,16], attempts to produce antiviral peptide or peptidomimetic substances that inhibit Tat binding to the TAR element were undertaken by a number of researchers [19–21]. A synthetic peptide/peptoid compound, which effectively inhibits Tat binding to the TAR RNA by competing for the Tat-binding site, was found using a combinatory approach by screening an oligomer peptide/peptoid library of 3.2 million entities. This compound, CGP64222, selected by the criterion of in vitro inhibition of the Tat-TAR interaction, displayed an efficient suppression of HIV replication in a cell culture [21].

Among natural RNA-binding molecules, aminoglycoside antibiotics have interesting properties that make them similar to peptide RNA-binders. They are known to bind efficiently to RNA structures, such as 16S RNA or introns type I [22]. Neomycin B, tobramycin and lividomycin A inhibit the HIV Rev-RRE interaction in vitro at concentrations of 1–10 μM, whereas kanamycin, gentamicin, streptomycin etc. do not display any inhibition at 10–100 μM. None of the aminoglycoside antibiotics inhibits the Tat-TAR interaction at concentrations below 100 μM [23]. Recently, it was shown that neomycin B inhibits the Tat-TAR interaction non-competitively in vitro in the range of 100 μM–1 mM. This phenomenon was attributed to the antibiotic association with the TAR RNA in the lower stem [24].

The above allows us to propose that an anti-HIV compound, which would suppress viral replication by inhibiting transactivation by Tat, may be prepared by combining a carbohydrate skeleton similar to aminoglycoside antibiotics with

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PII: S0014-5793(99)00092-7

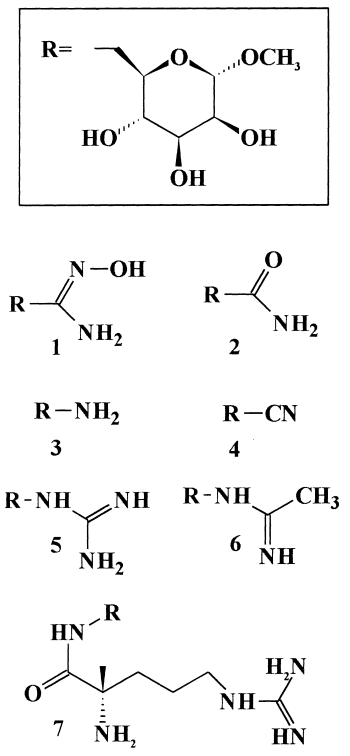

Fig. 1. The structures of 6-substituted MMP derivatives. Methyl 6-deoxy-α-D-mannoheptopyranuronic acid amidoxime (6-amideoximo MMP, **1**); methyl 6-deoxy-α-D-mannoheptopyranuronic acid amide (6-amido MMP, **2**); methyl 6-deoxy-6-amino-α-D-mannopyranoside (6-amino MMP, **3**); methyl 6-deoxy-6-cyano-α-D-mannopyranoside (6-cyano MMP, **4**); methyl 6-deoxy-6-guanidino-α-D-mannopyranoside (6-guanidino MMP, **5**); methyl 6-deoxy-6-(*N*-acetamidino)-α-D-mannopyranoside (6-(*N*-acetamidino) MMP, **6**); methyl 6-deoxy-6-(*N*-L-arginineamido)-α-D-mannopyranoside (RMMP, **7**). The synthesis and characterization of these compounds are presented in [25].

side chains of variable length bearing a guanidine moiety or a chemical group with a similar geometry or/and charge properties, resembling peptide side chains. Here we describe the discovery and preliminary studies of a new class of peptidomimetic TAR RNA binders: conjugates of arginine with aminoglycoside antibiotics. These relatively low molecular weight compounds are able to reproduce the binding behavior of the Tat protein and are promising candidates for antiviral drug design.

2. Materials and methods

2.1. Compounds

The synthesis and purification of the monosaccharide derivatives (Fig. 1) and aminoglycoside-arginine conjugates (Fig. 2) and their structures, confirmed by ^{1}H, ^{13}C NMR, 2D NMR and FAB MS, have been recently described [25].

2.2. TAR RNA and Tat peptide preparation

A 31 nucleotide TAR RNA fragment (5′-GGC CAG AUC UGA GCC UGG GAG CUC UCU GGC C-3′) containing the sequence 18–44 of the HIV-1 LTR [10] was transcribed in vitro by T7 RNA polymerase (Promega) from a synthetic single strand DNA template containing the 17 nucleotide double-stranded T7 promoter (both DNA oligonucleotides were prepared by the Weizmann Institute Chemical Services) [26]. The RNA was transcriptionally labelled with [α-^{32}P]UTP, purified on a 12% polyacrylamide/7 M urea gel (19:1 acrylamide:bis-acrylamide) and was eluted from the gel by extraction buffer, containing 0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA and 0.1% SDS. The sample was phenol-extracted and the RNA was precipitated from 70% ethanol. Purified RNA was dissolved in diethyl pyrocarbonate-treated water, its concentration was determined by UV absorption at 260 nm and the specific radioactivity was determined by scintillation counting on a LS 1701 Bruker counter.

The model Tat peptide (Tat R52: YKKKRKKKKKA) was prepared by the Weizmann Institute Chemical Services and was purified by reverse phase HPLC (C-18) [26].

2.3. Gel-shift assay

The binding reaction mixtures (20 μl) contained ^{32}P-labelled TAR (18–44) RNA oligonucleotide and the Tat R52 peptide in a binding buffer (10 mM Tris-HCl pH 7.5, 70 mM NaCl, 0.2 mM EDTA, 5% glycerol) as described [3,8,26]. The reaction mixtures were incubated for 10 min on ice and resolved by electrophoresis on a 10% non-denaturing PAG (40:1 acrylamide:bis-acrylamide) at 200 V, for 3 h at 4°C. Gels were dried and visualized by autoradiography. Quantitations were obtained by optical densitometry of the films. Different concentrations of TAR RNA (6, 12 and 20 nM) were titrated with various concentrations of Tat R52 in the binding reactions. CD_{50} values were defined as the Tat peptide concentration that displayed 50% binding to TAR RNA.

Binding inhibition by the monosaccharide derivatives was measured by adding several concentrations of each compound to reaction mixtures containing 60 nM Tat R52 and 6 nM ^{32}P-TAR, attaining 100% binding of Tat-TAR in the absence of inhibitors [26]. The binding inhibition values, CI_{50}, were defined as inhibitor concentrations that displayed 50% inhibition of Tat-TAR binding.

Binding of aminoglycoside-arginine conjugates to TAR RNA was measured by adding varying concentrations of each conjugate to reaction mixtures (20 μl) which contained 12 nM ^{32}P-labelled TAR RNA. The reactions were analyzed as described above. CD_{50} values were defined as the conjugate concentrations that displayed 50% binding to TAR RNA.

2.4. Affinity chromatography on L-arginine-Amberlite column

An L-arginine-Amberlite resin for affinity chromatography was prepared using the following procedure: ethanolamine was coupled to the carboxyl groups of Amberlite IRC-50 using 1-(3-dimethylaminopropyl) 3-ethylcarbodiimide, a water soluble coupler. The hydroxy ethylene amide resin obtained was washed and dried and L-arginine was coupled to the hydroxy groups using a standard cyanogen bromide protocol. The final concentration of arginine residues was 12–16 μM/ml resin. The swollen L-arginine-Amberlite resin (200 μl) was packed into a plastic mini-column and equilibrated with buffer containing 40 mM Tris-HCl, pH 7.5, 250 mM NaCl, 0.5 mM EDTA and 5 mM $MgCl_2$ (buffer A). Approximately 1 ng of ^{32}P-TAR RNA in buffer A was loaded on the column. The column was eluted 3–5 times with 400 μl portions of buffer A, until no radioactivity was detected in the eluent. The total radioactivity in the eluents did not exceed 3% of the total radioactivity loaded. Each of the tested compounds was dissolved in buffer A and passed through the column in 300 μl portions. Radioactivity of the eluates was determined by scintillation counting on a LS 1701 Bruker counter. CE_{50} values, the concentrations of inhibitors eluting 50% of the radioactive material from the column, were determined. The eluate samples were proven to contain TAR RNA by electrophoresis on a denaturing 12% polyacrylamide/7 M urea gel, followed by autoradiography.

The L-arginine-Amberlite affinity resin, described above, was found to be more mechanically stable and of better affinity properties than the L-arginine-agarose previously used (e.g. [8]). This may be due to the higher arginine concentration and 6-atom spacer between the polymer and the arginine residues.

2.5. Cytotoxicity test

The cytotoxicity of the compounds was studied in LAN 1 (human neuroblastoma), MPC11 (murine plasmacytoma) and MT4 (human T-lymphocytes) cell cultures. Approximately 2.5×10^{4} cells were seeded per each well of the standard 96-well Falcon tissue cultural plate in 200 μl of DMEM containing 10% of heat-inactivated fetal calf serum (FCS). Different concentrations of the compounds were added to the wells in triplicates. After 24–48 h incubation, the cells were washed and the medium was changed for the same amount of DMEM/10% FCS containing 0.5 μCi of [^{3}H]thymidine per well for 1 h. Then the medium was removed, the cells were washed with saline

and the DNA was precipitated by ice-cold 10% trichloroacetic acid. The precipitate was solubilized in 50 μl 1 M NaOH, mixed with 4 ml of Ultima Gold scintillation liquid (Packard) and counted on a LS 1701 Bruker scintillation counter.

2.6. Cellular uptake using fluorescent probes

The fluorescent derivative of the aminoglycoside-arginine conjugate was prepared as described [27]. R4K was reacted with fluorescein isothiocyanate (FITC, Sigma) in water-methanol-dimethyl sulfoxide mixture for 24 h. FITC was added in 1:1 molar ratio which enabled only one random amino group of the conjugate to react with FITC. The fluorescein-labelled conjugate was purified by extraction with acetone and absolute alcohol.

Hippocampal neurons from rat puppies were grown over rat glia cells on polylysine-coated glass cover slides [28]. Human peripheral blood mononuclear cells (PBMCs) were separated from a fresh blood sample on a Ficoll gradient by the standard procedure. They were cultured on polylysine-coated glass cover slides in RPMI medium, containing 10% FCS, for 24 h. The cells were incubated in HEPES-buffered saline containing 0.1–1 mg/ml of R4K-fluorescein probe for 1.5–2 h. The slides were washed several times with saline and studied by confocal laser-scanning microscopy on an Axiovert 100M (Zeiss) microscope, using excitation at 488 nm (argon-ion laser) and emission detected in a band of 505–550 nm [28].

3. Results

3.1. Monosaccharide derivatives

We prepared a set of 6-substituted derivatives of methyl α-D-mannopyranoside (MMP) (Fig. 1) in order to determine which side chain attached to a monosaccharide moiety would display the highest affinity to TAR RNA. These compounds were tested for their affinity to TAR on an L-arginine-Amberlite column, and their potential inhibition of Tat-binding to TAR, using the gel-shift assay. We observed that 6-guanidino 6-deoxy MMP (**5**) and 6-(*N*-acetamidino) 6-deoxy MMP (**6**) (Fig. 1) eluted ^{32}P-labelled TAR RNA from the column with CE_{50} of 650 mM and 730 mM, respectively, which is significantly lower than the CE_{50} of arginine (1500 mM). All the other tested monosaccharide derivatives displayed CE_{50} values of approximately 1500 mM or higher (Table 1). The gel-shift assays demonstrated that 6-guanidino MMP (**5**) and 6-(*N*-acetamidino) MMP (**6**) inhibited the binding of 60 nM Tat

Table 1
Concentrations of the compounds that eluted 50% of TAR RNA (CE_{50}) from the arginine-Amberlite affinity column

Compound	CE_{50} (mM)
R52 Tat peptide	0.5
Arginine HCl	1500
6-Amidoximo MMP (**1**)	>1500
6-Amido MMP (**2**)	>1500
6-Amino MMP (**3**)	>1500
6-Cyano MMP (**4**)	1200
6-Guanidino MMP (**5**)	650
6-(*N*-Acetamidino) MMP (**6**)	730
RMMP (**7**)	310
R4K	2.5
R3G mixture	1.4

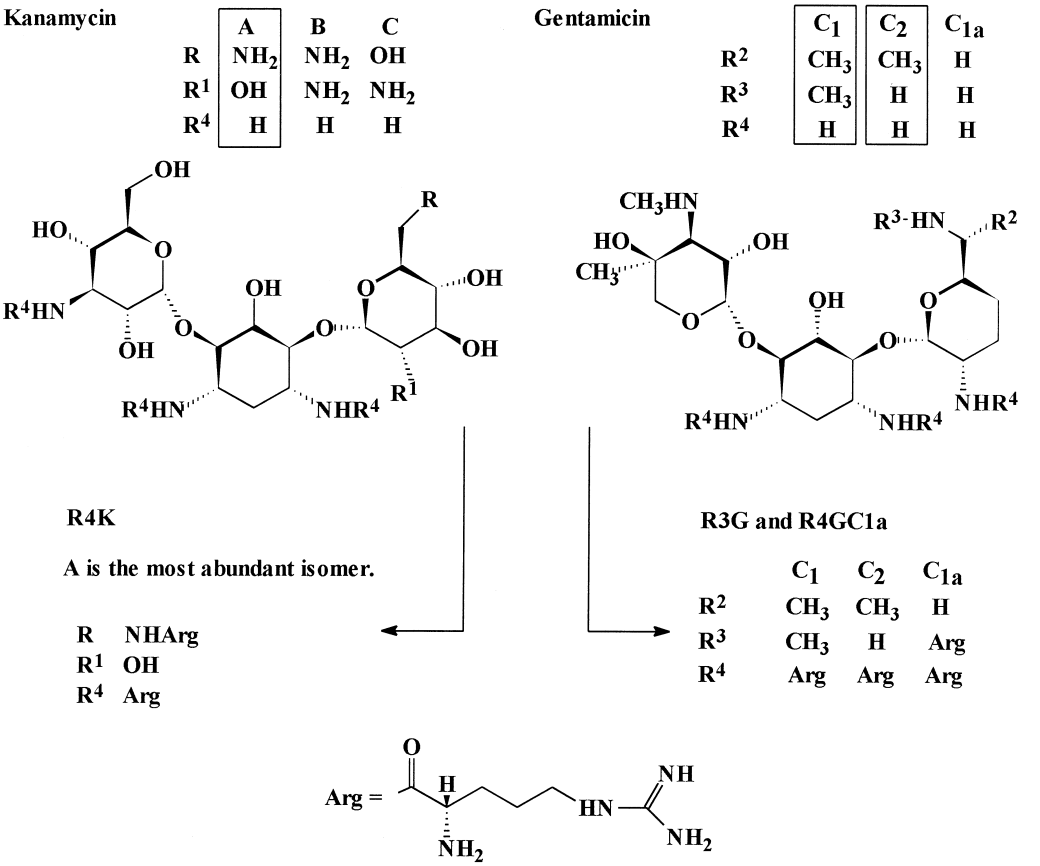

Fig. 2. The structures of kanamycin and gentamicin C conjugates with arginine. R4K is the major product (~90% in the mixture), the tetra-substituted derivative of the kanamycin A isomer. R3G is a mixture of tri-substituted derivatives of gentamicin C1 and C2 isomers. R4GC1a is a tetra-substituted derivative of the purified gentamicin C1a isomer.

R52 peptide to 6 nM TAR RNA with CI_{50} values of 9 and 11 mM, respectively. These CI_{50} values were normalized to the conditions presented by Frankel et al. [8], giving apparent K_i values of around 1 mM, suggesting that the 6-guanidino MMP (**5**) and 6-(*N*-acetamidino) MMP (**6**) are stronger inhibitors of Tat-TAR binding than the free arginine, and are similar to L-argininamide, agmatine [8], and neomycin B [23]. All other monosaccharide derivatives did not show significant inhibition of Tat binding to TAR (Table 2). These results indicate that a compound comprised of a carbohydrate 'core' attached to a single strongly basic group with the geometry resembling that of guanidine, may serve as a specific inhibitor of Tat binding to TAR.

The arginine-MMP conjugate (RMMP (**7**)) was prepared [25] and its affinity to TAR was determined as described above. We found that 310 mM of RMMP eluted 50% of the TAR RNA from the affinity column (Table 1). The gel-shift assay revealed that RMMP inhibited the Tat-TAR interaction with a CI_{50} of 1.8 mM which corresponds to an apparent K_i of approximately 160 μM (normalized as above [8]) (Fig. 3A and Table 2). Having compared the CI_{50} (or K_i) values of RMMP to the corresponding values of 6-guanidino MMP (**5**) and 6-(*N*-acetamidino) MMP (**6**), we concluded that not only a carbohydrate core facilitates the binding of the inhibitor, which contains a guanidinium (and acetamidinium) group, to TAR RNA, but a longer chain, connecting the core and the charge-bearing moiety, is also beneficial. Hence the 'best building block' for a potent antiviral peptidomimetic compound was considered to be a monosaccharide-arginine conjugate.

3.2. Aminoglycoside-arginine conjugates

In order to obtain oligomeric derivatives, we modified commercial aminoglycoside antibiotics, kanamycin and gentamicin C by attaching arginine moieties through amide bonds using standard peptide chemistry methods. In particular, conjugates of L-arginine with kanamycin, gentamicin C and gentamicin C1a were synthesized (Fig. 2 and [25]).

Kanamycin (Fluka) is a mixture of the three major compounds, kanamycins A, B and C, of which the A isomer was the most abundant (~80%). As expected, a kanamycin A derivative comprised approximately 90% of the arginine-kanamycin conjugate (R4K) mixture. ^{1}H and ^{13}C NMR spectroscopy, as well as 2D NMR studies, confirmed the purity and chemical structure of the conjugate which was found to have a 4:1 arginine to antibiotic ratio (Fig. 2). A mass peak of 1109.7 Da (calculated 1109.25 Da) was observed in FAB mass spectrum [25].

Gentamicin C (Nova-Biochem) is a mixture of three components, C1, C2 and C1a, that differ by methylation of a single amino group and adjacent CH_2 (Fig. 2) [29]. The components were separated chromatographically, as previously

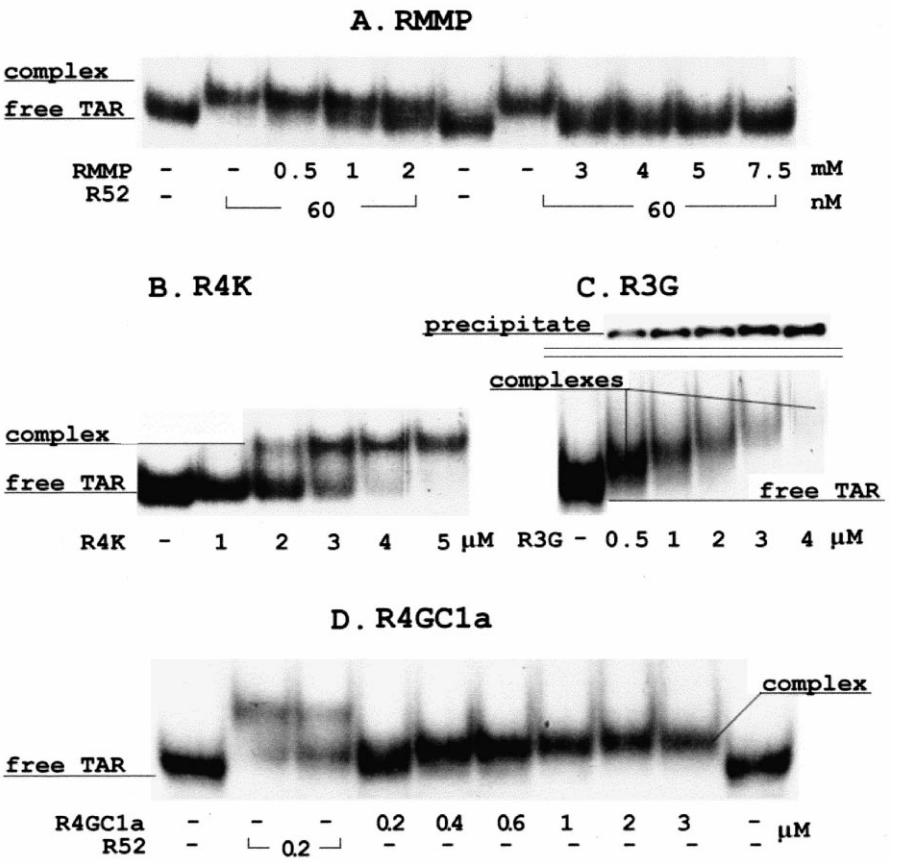

Fig. 3. Electrophoretic mobility shifts. A: Inhibition of Tat R52 peptide binding to TAR RNA by RMMP (**7**) (60 nM of Tat R52, 6 nM TAR). B: Binding of R4K to TAR RNA (12 nM). Supposed a 2:1 ratio of R4K to TAR RNA in the complex. C: Binding of the R3G mixture to TAR RNA (12 nM). Complexes of increasing molecular weight are observed and the RNA precipitation in wells is shown (the empty gel space between the horizontal lines is deleted). D: R4GC1a binding to TAR RNA (20 nM). Supposed a 1:1 ratio of R4GC1a to TAR RNA in the complex. The concentration of Tat R52 in the control lanes is 200 nM.

Table 2
Concentrations of the compounds that displayed a 50% inhibition (CI_{50}) and a 50% binding (CD_{50}) in gel-shift experiments on TAR RNA

Compound	CI_{50} (mM)	CD_{50} (mM)	TAR RNA (nM)
Tat R52 peptide		6–12[a]	2[a]
Tat R52 peptide		35	6
Tat R52 peptide		75	12
Tat R52 peptide		200	20
Arginine HCl	4[b]		2[b]
Arginine HCl	38		6
6-Amidoximo MMP (**1**)	>50		6
6-Amido MMP (**2**)	>50		6
6-Amino MMP (**3**)	56		6
6-Cyano MMP (**4**)	46		6
6-Guanidino MMP (**5**)	9		6
6-(*N*-Acetamidino) MMP (**6**)	11		6
RMMP (**7**)	1.8		6
R4K		2500	12
R3G mixture		500	12
R4GC1a		200	20

[a]Reference [3].
[b]Reference [8].

described [29]. The ratio of the isomers was 4:3:1 [25]. The arginine-gentamicin conjugate mixture (R3G) contained mainly species of three arginine residues attached to the antibiotic core (Fig. 2), as was confirmed by ^{1}H and ^{13}C NMR. FAB MS revealed two prominent mass peaks of 948.3 and 934.3 Da (calculated 948.1 and 934.1 Da), which correspond to tri-arginine derivatives of the C1 and C2 isomers, the major components of the mixture. The R4GC1a conjugate of arginine with pure C1a gentamicin isomer was also prepared. This conjugate comprised essentially one product, which contained four arginine residues (Fig. 2), according to ^{1}H and ^{13}C NMR [25].

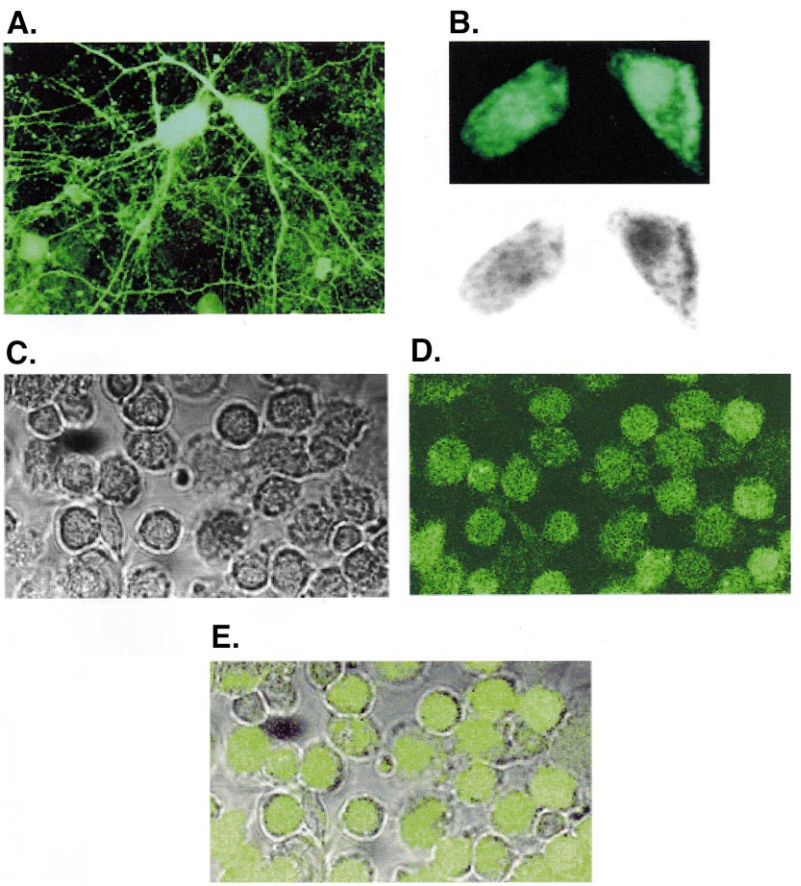

Fig. 4. Confocal microscopy images of live cells stained with R4K-FITC. Images were taken with the 63× water immersion objective. A: Rat hippocampal neurons incubated with 1 mg/ml R4K-FITC for 2 h. B: The same two cells at a higher magnification and contrast: an intense fluorescence is observed in the nuclei. C: Optical microscopy of human PBMCs. D: The same field, confocal microscopy at 488 nm excitation. The fluorescence is observed. E: Superposition of images C and D. Fluorescence is localized in the nuclei.

3.3. Binding of aminoglycoside-arginine conjugates to TAR RNA

The ability of aminoglycoside-arginine conjugates to bind TAR RNA was studied using the L-arginine-Amberlite affinity column. We found that the concentrations of R4K and R3G conjugates needed to elute 50% of the labelled TAR from the column (CE_{50} values of 2.5 mM and 1.4 mM, respectively, Table 1) were comparable to that obtained for Tat R52 peptide (CE_{50} value of 0.5 mM, Table 1). This result suggests that the aminoglycoside-arginine conjugates bind to the RNA with similar efficiency as Tat peptide and compete for the same arginine-binding site on TAR.

The molecular weights of the aminoglycoside-arginine conjugates (about 940, 1076 and 1109 Da for R3G, R4GC1a and R4K, respectively) are close to that of the Tat R52 peptide (1434 Da), thus their binding to TAR RNA was clearly observed as electrophoretic band shifts (Fig. 3). All the conjugates displayed a high affinity to TAR RNA, the binding of R4K to 12 nM ^{32}P-labelled TAR was observed with a CD_{50} of 2.5 μM (Fig. 3B, Table 2). The band shifts observed for the R4K complex with TAR remained unchanged at increasing concentrations of R4K in the range of 1–5 μM. Since a Tat-TAR complex has a 1:1 stoichiometry and the band shift of the R4K-TAR complex was observed to be approximately twice that of Tat R52-TAR (Fig. 3B), we suggested a 2:1 ratio of the R4K conjugate to RNA in the complex. The R3G conjugate displayed binding to 12 nM of TAR with a CD_{50} of about 500 nM (Fig. 3C, Table 2). The gel mobility of R3G-TAR complexes declined significantly with the increase of R3G concentration in the range of 0.5–4 μM, as was observed from the increase of the band shifts and an increasing precipitation of the complexes in the wells was observed (Fig. 3C). At the same concentration of TAR (12 μM), the CD_{50} of Tat R52 was found to be 75 nM (Table 2). The R4GC1a conjugate formed a complex with 20 nM of TAR RNA at concentrations starting from 200 nM. The CD_{50} of Tat R52 at the same concentration of TAR RNA was 200 nM. By comparing the gel shift of R4GC1a-TAR to that of Tat R52-TAR, we suggested that the ratio of R4GC1a to the RNA in the complex is 1:1 (Fig. 3D, Table 2). An approximate linear dependence of Tat R52 CD_{50} values on TAR RNA concentrations was observed (Table 2). We have normalized the CD_{50} values, obtained in our study, to the reported conditions [3], and apparent K_d values, suitable for comparison with the reported data, were produced. The apparent K_d values for the conjugate complexes with TAR RNA were found to be 416 nM for R4K, 83 nM for R3G and approximately 20 nM for R4C1a (within less than 20% estimated error). Thus, a tri-saccharide core bearing three or four arginine residues can successfully mimic the binding pattern of Tat.

3.4. Cytotoxicity test

All the compounds were tested for their toxicity in at least two of the three above mentioned cell culture lines. We found that none of the compounds up to a concentration of 1 mM caused any cell growth inhibition, as it was measured by [^{3}H]thymidine incorporation.

3.5. Cellular uptake using fluorescent probes

Uptake of the conjugate compounds by cells and intracellular distribution was studied using a fluorescent derivative of R4K (R4K-FITC).

After incubation of live rat hippocampal neurons with 1 mg/ml R4K-FITC for 2 h, an intense fluorescence was observed in the cells, indicating an efficient uptake of the probe (Fig. 4A). Even higher fluorescence was detected in the cell nuclei, suggesting that the R4K-fluorescein is associated with nucleic acids, as was expected (Fig. 4B). Live human PBMCs were treated with 0.1 mg/ml of R4K-FITC for 1.5 h. The accumulation of the fluorescent probe was observed in the cell nuclei (Fig. 4C,D,E).

4. Discussion

The conjugates of aminoglycoside antibiotics with arginine comprise a completely new class of peptidomimetic substances. The aminoglycoside-arginine conjugates resemble oligocationic peptides by their chemical properties, but it is not likely that they would be recognized by intracellular proteolytic enzymes. Conjugate structures share features of relative rigidity of the pyranoside sugar rings, with relative flexibility of inter-ring and side chain links. It is clear that guanidinium groups are important for the ligand-TAR interaction. General features of the ligand molecule which provide groove binding also play a significant role in HIV and BIV Tat protein complexation with their respective TAR elements [10–12], as well as in the HIV Rev interaction with the RRE responsive structure [30]. Aminoglycoside antibiotics are known to bind in the RNA major groove: e.g. tobramycin binds in the stem-loop junction of the RNA aptamer [31] and parmomycin and neomycin bind in the A-site of 16S RNA [32,33]. We suggest that the aminoglycoside-arginine conjugates, presented in this study, can bind in the TAR RNA major groove. Arginine side chains probably interact with nucleotides in the bulge region, while the glycoside core provides additional ligand-RNA contacts near the TAR loop.

Based on our study, we can propose some structural features of aminoglycoside derivative binding to TAR. As we observed, a sugar ring facilitates binding of the compound to the RNA, especially when the ring and the guanidinium groups are separated by a spacer of suitable length. Thus, 6-guanidino MMP (**5**) displayed six-fold lower activity then RMMP (**7**). It is likely that the guanidinium group can be substituted for the acetamidinium one, since the K_i values of 6-guanidino MMP (**5**) and 6-(*N*-acetamidino) MMP (**6**) are similar. It is worth noting that streptomycin, bearing two guanidino groups on the deoxystreptamine ring is not an efficient inhibitor of the peptide-RNA interaction [23]. So far L-arginine seems to be the best 'building block', but it is not necessarily the only one. Conjugates, bearing side chains of varied lengths with terminal guanidine or acetamidine groups, may also prove to be potent TAR RNA binders. Such conjugates can be prepared e.g. by modification of the terminal amino group of lysines or β-alanines (four or two methylene groups in the side chain respectively).

Arginine-saccharide oligomers bind TAR RNA significantly stronger than monomers. It is enough to recruit only four arginine residues to reach nanomolar K_d values. It is worth noting that the conjugate containing three arginine residues per molecule (R3G) binds RNA five-fold more efficiently than the conjugate with four arginine residues per molecule (R4K). This observation suggests that the geometry of the conjugate is ultimately more important than the number of arginine side chains. Since all the gentamicin derivatives contain an un-

modified amino sugar ring (Fig. 2), it is plausible that such a ring favors groove binding near the TAR RNA loop, similar to tobramycin ring III [31]. The R4GC1a conjugate, which contains an unmodified amino sugar ring and four arginine residues, binds to TAR RNA almost as efficiently as the Tat R52 peptide does. The conjugates form different complexes with the RNA, as was observed from their band shifts. We assume that R4K and R4GC1a conjugates form soluble complexes with the RNA at 2:1 and 1:1 ligand to RNA ratio, respectively. The R3G conjugate mixture forms complexes of varying stoichiometry, and precipitates the RNA at relatively low concentrations, probably due to the formation of branched agglomerates. Structure-functional relationship of the conjugate-TAR RNA interactions will be further investigated by footprinting analysis, NMR structural studies and by X-ray crystallography.

Fluorescent labelling studies indicate that the conjugates are subject to intracellular transport. Preferentially intranuclear accumulation suggests their general affinity to nucleic acids.

In conclusion, the aminoglycoside-arginine conjugates, R4K, R3G and R4GC1a, displayed a high affinity to the target TAR RNA and were found to be non-toxic for cultured cells even at relatively high concentrations. The synthesis of the conjugates utilizes naturally occurring antibiotics and requires fewer steps than peptide or peptoid preparation. There is strong evidence that the conjugates penetrate into live cells. All these features make aminoglycoside-arginine conjugates attractive candidates for antiretroviral drug design.

Acknowledgements: A. Evdokimov and A. Litovchick are doctoral fellows of the Feinberg Graduate School of the Weizmann Institute of Science (Israel). This work was supported in part by research grants from Yeda Research Co. (WIS) and the Israel Ministry of Science to Prof. A. Lapidot.

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