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INHIBITION OF AN HIV-1 TAT-DERIVED PEPTIDE BINDING TO TAR RNA BY AMINOGLYCOSIDE ANTIBIOTICS

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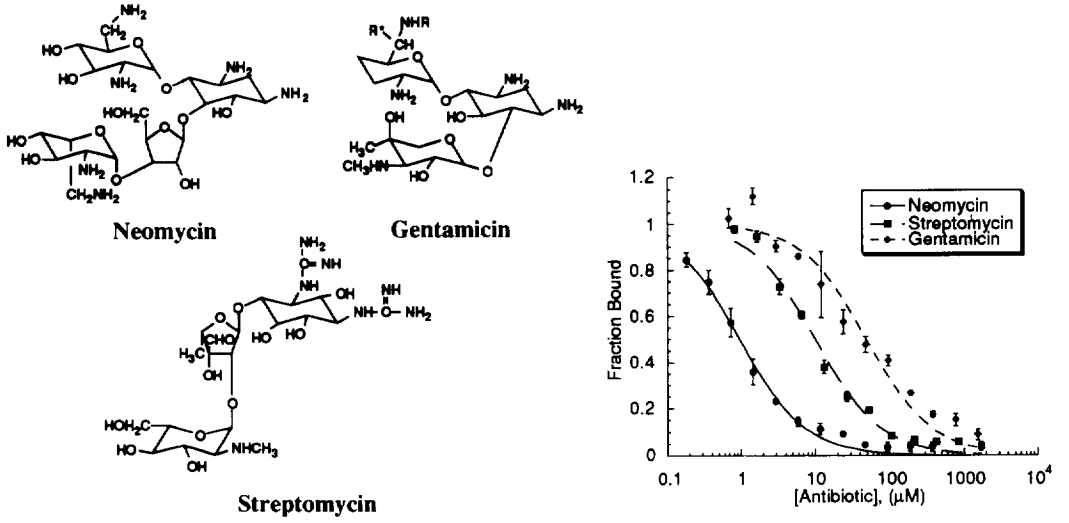
Abstract: Aminoglycoside antibiotics were found for the first time to inhibit an HIV-1 Tat-derived peptide binding to TAR RNA. The IC_{50} values of neomycin, streptomycin, and gentamicin were determined as 0.92 μM, 9.5 μM, and 45 μM, respectively. In the absence of Tat peptide these antibiotics were found to cause mobility shifts of the TAR RNA on non-denaturing polyacrylamide gels. This is the first example, to our knowledge, of non-peptide or non-nucleotide like small molecules that bind to and induce a mobility shift of TAR RNA. It was further found that the antibiotics which demonstrate higher affinity for TAR are better inhibitors of the Tat/TAR interaction. Mutational and competition studies indicate that neomycin binds to the duplex domain of TAR RNA.

Tat, an 86 amino acid regulatory protein of HIV-1, contains an arginine rich basic domain (residues 47 to 58) involved in binding to TAR RNA (residues 1-59 of HIV-1 mRNA).[1] TAR adopts a stable hairpin-stem-bulge structure.[2] A direct correlation has been found between Tat binding to TAR RNA and trans-activation mediated by Tat.[3] Certain aminoglycoside antibiotics have been found to interact with ribosomal RNAs.[4] More recently, aminoglycosides such as streptomycin and neomycin have been demonstrated to bind to *Tetrahymena* intron I RNA[5] and to the Rev response element (RRE) region of HIV-1 mRNA.[6] The binding of the antibiotics subsequently inhibits the functions of these RNAs. We report here that certain aminoglycoside antibiotics bind to TAR RNA and inhibit its association with a peptide derived from Tat protein.

Peptides with sequences derived from the Tat protein have been used previously to model the interaction of full length Tat protein and its cognate RNA.[7] We have synthesized a 12-amino acid peptide (Tat_{12}) and a 40-amino acid (Tat_{40}) peptide, both consisting of the TAR-binding domain of the Tat protein.[8] The interaction of Tat_{12} or Tat_{40} with a 31-nucleotide RNA, TAR_{31}, containing residues 18 to 44 of the native TAR RNA,[9] in the absence or presence of aminoglycosides were followed using gel electrophoresis.[10] After electrophoresis, the relative amounts of free and bound TAR_{31} were used to determine the apparent K_d of the peptide[11] or the IC_{50} values of the added aminoglycoside antibiotics.

Preliminary studies demonstrated that a wide variety of aminoglycosides inhibit Tat_{12} binding to TAR_{31}.[12] Neomycin (a 4, 5-disubstituted aminocyclitol), gentamicin (a 4,6- disubstituted aminocyclitol), and streptomycin (a guanidine derivatized aminocyclitol) were selected for more detailed studies using Tat_{40}. The structures and



(A) (B)

Figure 1. (A) Chemical structures of neomycin B, streptomycin, and gentamicin. Gentamicin obtained from Sigma is a mixture of gentamicin C_1 (R=CH_3, R*=CH_3), C_2 (R=H, R*=CH_3), and C_{1A} (R, R*=H). (B) Inhibition of Tat_{40} peptide binding to TAR_{31} by aminoglycosides. Fraction bound (Tat_{40}/TAR_{31} complexes) are plotted against the added concentration of antibiotic (μM in logarithmic scale).

inhibition data of these antibiotics are shown in Figure 1.[13] Neomycin exhibits an IC_{50} of 0.92±0.09 μM[14] while the IC_{50} values of streptomycin and gentamicin are 9.5±0.8 μM and 45±4 μM, respectively. Apparently, the IC_{50} values are not a simple function of the number of positive charges contained in these small molecules.[15] Streptomycin (+3 charge) is 10-fold less effective than neomycin (+6 charge) but is about 5-fold more effective than the more cationic gentamicin (+5 charge) in inhibiting Tat_{40} binding. A complete structure-activity study of the aminoglycoside antibiotics is required to demonstrate what properties (electrostatic and/or structural) affect the inhibitory activity of these molecules.

To understand the nature of the aminoglycoside antibiotic inhibition of Tat_{40} binding to TAR_{31}, the direct interaction of the antibiotics with TAR_{31} in the absence of Tat_{40} was evaluated. Aminoglycosides, due to their polycationic properties, are anticipated to interact with polyanionic RNA or with the Tat_{40}/TAR_{31} complex rather than with the cationic Tat_{40}. This is supported by our observation of additional electrophoretic bands that do not correspond to either free or Tat_{40}-bound TAR_{31} in the inhibition studies with neomycin, streptomycin, or gentamicin.[16] Indeed, in the absence of Tat_{40} peptide, neomycin, streptomycin, or gentamicin interact with TAR_{31} and induce mobility shifts in TAR_{31}. The formation of antibiotic-TAR_{31} complexes as a function of drug

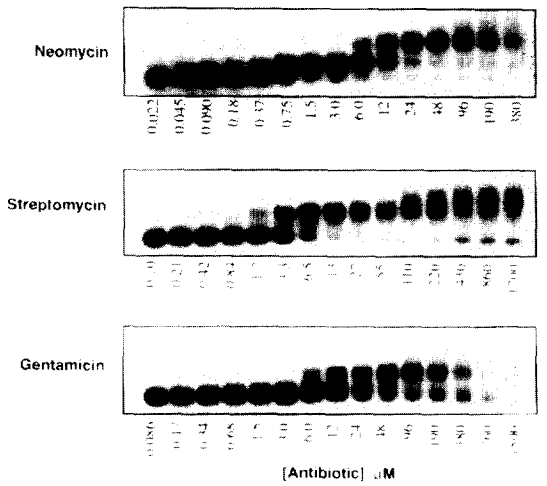


Figure 2. Direct binding of antibiotics to TAR_{31}. An estimated 100 pM of ^{32}P-TAR_{31} was titrated with known concentrations of neomycin, streptomycin, or gentamicin.

concentration are shown in Figure 2. To the best of our knowledge, this is a novel observation for RNA/aminoglycoside interactions of any type. Additionally, in the titration studies with each antibiotic, more than one bound species of TAR_{31} is observed. The higher-order complexes occur at higher antibiotic concentrations and subsequent to the formation of an initial drug/TAR_{31} complex.[17] The antibiotic/TAR_{31} complexes are stable to electrophoretic conditions and sometimes migrate with mobilities similar to those of Tat peptide/TAR complexes (in particular, when Tat_{12} is used [data not shown]).[18] By defining a measurement of binding affinity, C_{50} (a concentration at which 50% of the total RNA is bound by antibiotic), the antibiotics were found to complex to TAR_{31} in the following order: neomycin > streptomycin > gentamicin.[19] These results and the Tat_{40} inhibition data described above indicate that the better RNA binders (e. g., neomycin) demonstrate larger inhibition effects (smaller IC_{50} values) on Tat binding to TAR_{31}.[20]

Mutational and competition studies of TAR_{31} have demonstrated that the duplex stem region of TAR_{31} is involved in aminoglycoside recognition. Figure 3 shows the sequences and predicted secondary structures of TAR_{31} and TAR mutants.[21] The predicted secondary structures of the mutants were supported by an observation that a reduction of > 20-fold in Tat_{40} binding was found when the mutations were introduced at the bulge domain but little or no changes in Tat_{40} binding were found when the mutations were at the loop domain. Under similar conditions, the binding characteristics of neomycin and the C_{50} values obtained for TAR mutants are similar to that of TAR_{31} (shown in Figure 2). Mutations introduced at the UCU bulge or at the loop domain have little or no effect (with one exception) on neomycin binding.[22] Higher-order complexes or neomycin/TAR_{31} were found in all but one mutant, TAR_{31} with the loop residues replaced by a polyethyleneglycol linker. A competition study using a system containing neomycin, ^{32}P-labeled TAR_{31}, and un-labeled TAR mutants was also performed. Consistent with the direct binding experiments, all TAR mutants and TAR_{31} compete equally well the binding of ^{32}P-labeled TAR_{31} with neomycin. These results suggest that neomycin binds initially to the duplex domain of

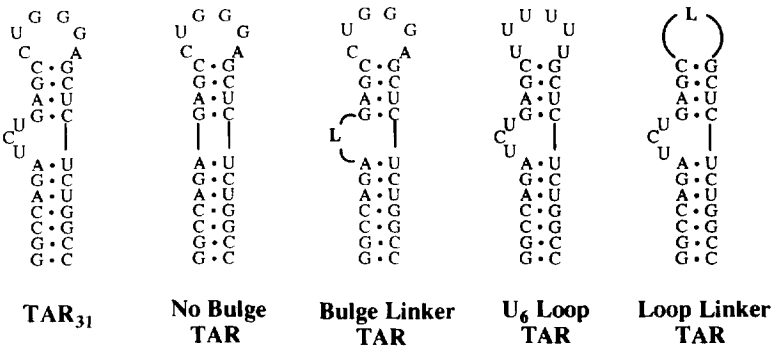


Figure 3. Sequences and proposed secdonary structures of TAR_{31} and TAR mutants. L = -O-[$(CH_2)_2$-O]$_4$-$(CH_2)_2$-O.

TAR_{31}, followed by a less-selective (or a cooperative) binding of neomycin to the loop domain. In addition, competition of ^{32}P-TAR_{31}/neomycin complexes by non-TAR like nucleic acids were found to follow the order of: double-stranded RNA (poly I • poly C) > yeast tRNA > double-stranded DNA (poly dA-dC • poly dG-dT) > single-stranded RNA (poly C). These results further support the binding selectivity of neomycin. Studies are underway to verify any sequence specificity of neomycin/TAR_{31} interaction.

In summary, we have demonstrated that a class of non-peptide, non-intercalative molecules, aminoglycoside antibiotics, inhibit Tat_{40} peptide binding to a portion of TAR at μM concentrations. This is, to our knowledge, the first example of small molecules that can form kinetically stable complexes with TAR RNA and subsequently inhibit Tat binding. These findings offer a unique opportunity to characterize, both qualitatively and quantitatively, the binding properties of TAR_{31} to three non-peptide small molecules, neomycin, streptomycin, and gentamicin. Further definition of the interactions between aminoglycoside antibiotics and TAR RNA will provide insight into the design of small molecule antagonists with specificity for this receptor RNA.

Supplementary Material Available: Experimental details and methods of data analysis to obtain IC_{50} and C_{50} values reported here are available from the authors.

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8. Tat_{40}, YGRKKRRQRRRPPQGSQTHQVSLSKQPTSQPRGDPTGPKE, and Tat_{12}, YGRKKRRQRRRG, were synthesized by solid phase synthesis using standard Boc chemistry protocols, purified by reversed-phase HPLC, and characterized by ES mass spectrometry (m.w. of Tat_{40}: calculated, 5663.0; found, 5663.0, and m.w. of Tat_{12}: calculated, 1616.9; found, 1617.6).
9. TAR_{31}, 5'-GGCCAGAUCUGAGCCUGGGAGCUCUCUGGCC-3', was chemically synthesized using phosphoramidite chemistry, purified by PAGE, and characterized by enzymatic sequencing. For all studies, TAR_{31} was 3'-end labeled with T4 RNA ligase (Pharmacia) using 5'-^{32}P pCp (Amersham) and reannealed by heating to 90 °C before use for binding studies. All binding studies were performed in a buffer solution containing 10 mM Tris-HCl (pH 7.5), 70 mM NaCl, 0.2 mM EDTA, 0.01% Nonidet P-40 (Sigma), 5% glycerol. The RNA mobility-shift assays were performed on 1.5-mm thick, non-denaturing gels (15% or 20% acrylamide with 1/75 of bis).
10. Tao, J. and Frankel, A.L. *Proc. Natl . Acad. Sci. USA* **1992**, *89*, 2723. Gel mobility shift assay was used to determine that the inhibition constant, K_i, for L-arginine was 4 mM, fot L-argininamide was 1 mM in inhibiting a Tat peptide (residues 49 to 57) binding to TAR RNA.
11. Under our experimental conditions, an apparent K_d of 0.2 nM was determined for Tat_{40}. This value is similar to previously reported K_d's for other Tat-derived peptides.[7]
12. At 3 mM concentration, neomycin, tobramycin, paromamycin, lividomycin, kanamycin, benkamycin, gentamicin, sisomicin, and streptomycin inhibit Tat_{12} peptides binding to TAR RNA. Under the same conditions, L-arginine, spermidine, and non-aminocyclitol antibiotics such as kasugamycin and spectinomycin had little or no effect in inhibiting Tat_{12} binding.
13. IC_{50} values were determined as follows: A pre-formed Tat_{40}/TAR_{31} complex (≈ 100 pM ^{32}P-TAR_{31} and 0.5 nM Tat_{40}) was challenged with varying concentrations of aminoglycosides. The mixtures were equilibrated for 15-20 min before submitted to gel electrophoresis. All reactions and electrophoresis were performed at room temperature (≈ 20 °C). For each aminoglycoside studied 12 concentrations were chosen, evenly distributed about the approximate IC_{50} value. Each data point was run in triplicate along with 4 control reactions (no aminoglycoside) on each gel. A total of 3 or 4 independant determinations of IC_{50} for each aminoglycoside were performed.

14. Neomycin has been previously reported to be a specific inhibitor for the Rev-RRE interaction and not active in preventing Tat peptide (residues 48 to 60) binding to a portion of TAR RNA (residues 18 to 44) at 100 μM concentration.[6] Compared to the neomycin data reported here, the discrepancy might result from the use of a different peptide sequence or different experimental conditions. For example, in the presence of 100 μg of yeast tRNA (non-specific carrier RNA), no binding of either Tat_{40} or neomycin to TAR_{31} was observed.
15. Botto, R. E. and Coxon, B. *J. Amer. Chem. Soc.* **1983**, *105*, 1021. Under pH 7.5 buffered conditions, neomycin is reasonably assumed to have a net +6 charge, gentamicin should have a +5 charge, while streptomycin should carry a +3 charge.
16. Of the antibiotics examined in our initial inhibition studies,[12] only neomycin, streptomycin, and gentamicin induced any mobility shifts in TAR_{31}.
17. These drug/TAR_{31} bands indicate the formation of non-stoichiometric complexes between the antibiotic and TAR_{31}. The molecular details of these antibiotics' interactions with TAR RNA are the subject of current investigation.
18. Based on the same observation, this also suggests that caution should be taken in interpretating the gel mobility shift data of Tat/TAR inhibition by aminoglycosides.
19. The accurate determination of C_{50} was made difficult owing to experimental details of the gel techniques. Even though the gel data routinely proceeded to only 85-90% of the theoretical saturation level, the assumption was made to utilize the standard binding equation to determine the 50% binding level based on a binding curve which does go to saturation. At the present time we are unable to account for the fact that binding of the aminoglycosides does not go to saturation. Although this is not a uncommon phenomenon that is found with complexes of only moderate stability (K_d in μM range). The mean of the values obtained with their standard deviations for neomycin, streptomycin and gentamicin were 0.7±0.2, 2.4±0.2, and 4.3±0.9 μM, respectively.
20. Due to the error inherent in these measurements, it is much more significant that IC_{50} and C_{50} results are of similar magnitudes than that C_{50} values are uniformly lower than IC_{50} values. Compared with footprinting-type experiments which were usually performed at ≈ 0.1 mM concentrations of aminoglycoside, these data provide strong circumstantial evidence that the antibiotics block Tat peptide from binding to TAR RNA by interacting with TAR RNA.
21. Similar TAR mutants have previously been used to determine the binding affinity and characteristics for Tat peptide.[23]
22. When neomycin was added greater than 1 μM, the reduction of the first neomycin/TAR complex was accompanied by increasing amounts of both the higher-order complex and free TAR. Further investigation is underway to understand the nature of these antibiotic/TAR complexes.
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