Structural Rearrangements of HIV-1 Tat-responsive RNA upon Binding of Neomycin B*

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Binding of human immunodeficiency virus type 1 (HIV-1) transactivator (Tat) protein to Tat-responsive RNA (TAR) is essential for viral replication and is considered a promising starting point for the design of anti-HIV drugs. NMR spectroscopy indicated that the aminoglycosides neomycin B and ribostamycin bind to TAR and that neomycin is able to inhibit Tat binding to TAR. The solution structure of the neomycin-bound TAR has been determined by NMR spectroscopy. Chemical shift mapping and intermolecular nuclear Overhauser effects define the binding region of the aminoglycosides on TAR and give strong evidence for minor groove binding. Based on nuclear Overhauser effect–derived intermolecular distance restraints, a model structure of the TAR-neomycin complex was calculated. Neomycin is bound in a binding pocket formed by the minor groove of the lower stem and the uridine-rich bulge of TAR, which adopts a conformation different from those known. The neamine core of the aminoglycoside (rings I and II) is covered with the bulge, explaining the inhibition of Tat by an allosteric mechanism. Neomycin reduces the volume of the major groove in which Tat is bound and thus impedes essential protein–RNA contacts.

Antibiotics are chemicals that are active against microorganisms, exerting their function in different ways at various cellular locations. Aminoglycoside antibiotics, for example, target the 30 S subunit of ribosomal RNA and cause mistranslation. Molecules of the neomycin family of aminoglycosides (Fig. 1a) bind directly to the A site of 16 S ribosomal RNA (1) and efficiently disturb protein biosynthesis of prokaryotes. Structural studies on the interaction of aminoglycosides with RNAs provided insights into the mechanisms of miscoding (2–4). The antibiotic distorts the structure of the RNA and thus leads to errors in protein biosynthesis. Variations in eukaryotic ribosomal RNA prevent high affinity binding of aminoglycosides to the ribosomes of higher organisms, making them less prone to antibiotic influence and thus rendering the antibiotics valuable medical drugs. Due to the growing problem of antibiotic resistance, caused by only a small number of mutations in the microorganisms, the determinants for antibiotic binding to RNA are of major interest in structural biology. Only a few structures of antibiotic–RNA complexes have been determined experimentally to date, among them the structure of paromomycin in complex with a model oligonucleotide comprising the A site of 16 S rRNA (2) and a low resolution and two high resolution structures of complexes between RNA aptamers and tobramycin or neomycin (5–7).

Aminoglycosides have also been found to bind to group I introns (8), to hammerhead RNA (9), and to human hepatitis δ virus ribozymes (10). These antibiotics also bind to the Rev (regulator of expression of the virophage) and Tat (transactivator of transcription) binding regions of human immunodeficiency virus type 1 (HIV-1)1 RNA, Rev response element (11), and Tat-responsive element (TAR) (12). Different modeling approaches yielded several structural models of complexes between neomycin and group I intron (13), hammerhead ribozyme (14), and Rev response element (15). In all of these complexes, the aminoglycoside is bound in the major groove of the RNA duplex at positions where the regular A-helical geometry is distorted by internal loops, bulged out nucleotides, or nonregular base pairs. No additional common structural features of the RNAs are evident. In contrast, the three aminoglycoside–RNA aptamer complexes show a bulged out nucleotide that flaps over the antibiotic forming a binding cavity, whereas the major groove of the A site RNA, favored by an internal loop, is simply widened upon paromomycin binding (2, 3).

Studies of the HIV-1 TAR RNA–neomycin interaction revealed that TAR also undergoes a conformational change upon antibiotic binding (16). TAR forms a base-paired stem closed by an apical loop (Fig. 1b). A triple-U bulge interrupts the stem only four base pairs below the loop and divides it into a lower and an upper part. The binding region of neomycin has been identified in the bulge and lower stem region, and competition experiments have shown that neomycin inhibits the HIV-1 Tat binding to TAR (16). Tat is an essential transcription factor for viral replication that binds to the human cellular protein cyclin T1 and to the stem-loop structure of TAR (17). The ternary complex of human cyclin T1, interacting with the loop, and Tat, interacting with the major groove in the bulge region of TAR, causes cyclin-dependent kinase 9 to hyperphosphorylate the COOH-terminal domain of RNA polymerase II, allowing efficient transcription of the viral genome. Inhibition of Tat binding to TAR represses recruitment of cyclin-dependent kinase 9, thus inhibiting phosphorylation of RNA polymerase II and rendering transcription nonprocessive. Thus, the Tat–TAR–human cyclin T1 interaction is an ideal target for drugs against HIV. Understanding of the binary interaction between Tat and

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TAR and their inhibition may serve as an initial step for studying the much more complicated ternary complex.

**MATERIALS AND METHODS**

Neomycin B sulfate was from Roth (Karlsruhe, Germany), ribostamycin sulfate was from ICN (Aurora, Ohio), and BP3 was chemically synthesized (German Cancer Research Institute, Heidelberg).

**RESULTS AND DISCUSSION**

Binding of both neomycin B and ribostamycin (see Fig. 1a) to HIV-1 TAR was monitored by one-dimensional NMR spectroscopy. Upon the gradual addition of either aminoglycoside to

![FIG. 1](image_url)  
**A**. aminoglycosides neomycin and ribostamycin are identical in the first three rings, and neomycin comprises an additional fourth ring. **B**, sequence and secondary structure of the 29-nucleotide TAR; open boxes indicate Watson-Crick base pairs.
The only satisfactory fit was achieved with the assumption of a 1:1 complex formation with either aminoglycoside, and the dissociation constants ($K_D$) are calculated by fitting the data to a one-binding site model. For TAR with ribostamycin two additional values with 5 and 10 equivalents of the antibiotic were also used for the fit.

The NMR imino proton spectrum of TAR in the presence of either aminoglycoside showed one set of resonances, demonstrating that free and aminoglycoside-bound TAR exchange in the intermediate to fast region of the NMR time scale.

Resonances indicating hydrogen bonding and formation of Watson-Crick base pairs are observed for all base pairs in the stem, except for A23-U47, which is located directly below the triple-U bulge. The most pronounced shifts of imino proton resonances are observed for G21, U24, and G26. The imino proton of G21 shows a resolved signal over the whole titration range. The neomycin-containing sample aggregated if more than three equivalents of the antibiotic were added to TAR. For ribostamycin, no aggregation was observed for ratios up to 10 equivalents of the aminoglycoside. An equation describing complex formation was fitted to the changes of the chemical shift of G21 H-1 (Fig. 2) as described under "Materials and Methods." The only satisfactory fit was achieved with the assumption of a 1:1 complex formation with either aminoglycoside, and the binding constant was calculated to be $5.9 \pm 2 \mu M$ for neomycin and $127 \pm 24 \mu M$ for ribostamycin. Due to the lack of values for higher excesses of neomycin, the uncertainty of the latter $K_D$ value was higher. This $K_D$ is of the same order as the $K_D$ of 0.92 $\mu M$ for neomycin complexed with the TAR 24U→C variant by gel retardation experiments (16).

**Neomycin Inhibits Tat Binding to TAR**—The Tat-derived peptides BP1 and BP1sw have been shown to have very similar properties with respect to TAR binding as full-length Tat (29, 30). The BP1sw-derived peptide BP3 with the amino acid sequence YHSQVWFTKGLGIRKKKQSLTSPQHGRHDPFIPKQI has been selected by phage display as a high affinity TAR-binding peptide (31). TAR in the presence of BP3 shows an imino proton spectrum (Fig. 3b) characteristic for a conformation that is also observed in the presence of BP1sw or other Tat-derived peptides (28, 32) and thus may indicate the Tat-bound conformation. After the addition of one equivalent of neomycin to the 1:1 TAR/BP3 sample, the spectrum of the imino protons of TAR changed (Fig. 3c) and strongly resembled the spectrum of TAR in the presence of neomycin alone (Fig. 3d), suggesting that neomycin changes the Tat-bound conformation of TAR. Compared with the corresponding spectrum in the presence of Tat peptides, a completely different resonance pattern is observed. The imino proton resonances of G21 shows a large upfield shift in the Tat-bound conformation. After the addition of neomycin, the resonance is downfield shifted as in the presence of neomycin alone. This clearly confirms competition experiments by gel retardation and CD spectroscopy (12, 16), indicating that the aminoglycoside is able to cause dissociation of Tat peptides from TAR. The results obtained at nano- and micromolar concentrations are thus also valid at millimolar concentrations, and, consequently, NMR spectroscopy is a suitable method to examine TAR-neomycin interaction.

**Fig. 2.** Titration of TAR RNA with neomycin (left) and ribostamycin (right). To a sample containing 200 $\mu M$ TAR RNA in 90 mM NaCl, 18 mM potassium phosphate, pH 6.4, H2O/D2O (9:1, v/v), each antibiotic was added from a 5.0 mM stock solution in the same buffer. Experiments were performed at 28 °C at 600 MHz. Shown is the change of chemical shift value ($\Delta$) of G21 H-1 versus the antibiotic/TAR ratio. Dissociation constants ($K_D$) were calculated by fitting the data to a one-binding site model. For TAR with ribostamycin two additional values with 5 and 10 equivalents of the antibiotic were also used for the fit.

**Fig. 4.** Two-dimensional NOESY spectrum of TAR in the presence of neomycin. The spectrum was recorded at 28 °C at 600 MHz with a mixing time of 150 ms. The sample contained 2.0 mM TAR and 4.0 mM neomycin in 90 mM NaCl, 18 mM potassium phosphate, pH 6.4, H2O/D2O (9:1, v/v). H1′H-6/H-8 chain tracing is shown from A20 through U31, skipping U23–U25. Between A20 and G26 an NOE is observed (black arrow), indicating proximity. Grey circles with arrows indicate the positions of cross-peaks in the spectrum of free TAR for which the largest shifts are observed upon the addition of neomycin; for U23 and U24, the H5/H-6 cross-resonance is shown.
BP3-TAR-neomycin samples with an excess of neomycin could not be prepared because of irreversible aggregation that occurred as soon as more than one equivalent of neomycin was added to the BP3-TAR sample. Thus, no further information on the kinetics of the competition could be deduced from our experiments.

Structure Determination—To further characterize the TAR-neomycin complex, we determined the neomycin-bound structure of TAR RNA in solution. For resonance assignment in two- and three-dimensional NMR spectra, we used unlabelled samples containing TAR and one or two equivalents of neomycin or ribostamycin and a 1:1 TAR-neomycin sample in which all adenosines, cytidines, and uridines were completely 13N/15C-labeled. In addition to standard homonuclear two-dimensional (NOESY, DQF-COSY, and TOCSY) and heteronuclear three-dimensional NMR experiments (13C HSQC-NOESY, HCCH-COSY, and HCCH-TOCSY), 13C-half-filtered experiments (NOESY and TOCSY) proved necessary for the assignment of the RNA resonances. In these experiments, the 13C magnetization is removed prior to the NOESY or TOCSY transfer, and thus only resonances from guanosine protons were observed in the partially labeled sample, rendering H-1′–H-8 resonance assignments of all guanosine protons possible. Sequential NOEs between protons from guanosines and protons from labeled nucleotides could be distinguished from cross-resonances overlapping in standard homonuclear spectra. Despite the strong degeneration of the ribose H-3′, H-4′, H-5′, and H-5′ resonances, 61% of the ribose resonances could be assigned unambiguously.

The strongest shifts (>0.2 ppm) upon aminoglycoside binding were observed for the H-5′–H-6 cross-resonances of U23 and U24 and for the H-1′–H-8 cross resonance of A22 in the presence of either antibiotic (Fig. 4). Mapping all shifts larger than 0.05 ppm renders the binding region for the antibiotics well localized; it spans from the bulge, including G26, to the lower stem down to base pair C19-G43. No resonance shifts are observed in the upper stem, except for G36, which is directly flanking the binding region, and for G28 H-8. This shift can be attributed to changes in the location of U25 caused by a new bulge conformation.

Most protons of ring I and ring IV are frequency-degenerated. If NOEs were observed with both neomycin and ribostamycin, NOEs were assigned to ring I protons. For G26 H-8, neither NOE was observed with ribostamycin; thus, it was assigned to ring IV protons.

| TAR proton | Neomycin proton | Distance interval |
|-----------|-----------------|------------------|
| G26 H-1′  | Ring I H2′ or H4′ | 4.0              |
| G26 H-1′  | Ring I H6′ or H6′ | 5.0              |
| G26 H-1′  | Ring I H3′      | 5.5              |
| U40 H-1′  | Ring I H2′ or H4′ | 5.5              |
| U40 H-1′  | Ring I H3′      | 6.5              |
| U40 H-1′  | Ring I H6′ or H6′ | 6.0              |
| G44 or U4′ H-1′ | Ring I H4′ or H2′ | 5.0              |
| G21 H-1′  | Ring II H3      | 6.5              |
| G21 H-8   | Ring III H1     | 7.5              |
| G21 H-8   | Ring III H3     | 7.5              |
| U23 H-5   | Ring I H2       | 5.5              |
| U23 H-1′  | Ring I H2′      | 4.5              |
| G43 H-1′  | Ring II H6      | 6.5              |
| G44 H-8   | Ring IV H3      | 6.5              |
| G44 H-8   | Ring IV H6′ or H6′ | 6.5       |

| Parameter | Value |
|-----------|-------|
| Average energy (kcal/mol) | -228.43 (±55.10) |
| E_{total} | 24.52 (±3.05) |
| E_{bond}  | 190.09 (±11.88) |
| E_{improper} | -346.21 (±27.00) |
| E_{improper} | -191.40 (±60.80) |
| E_{NOE} | 89.43 (±16.79) |
| E_{nhb} | 1.03 (±0.44) |
| Bond length | 0.004 |
| r.m.s. deviation from ideal angles (degrees) | Bond angles 0.92 |
| Improper angles 0.92 |
| Dihedral angles 0.10 |
| Heavy atom r.m.s. deviation for 17 lowest energy complex structures (Å) | RNA 2.28 |
| Neomycin 1.32 |
| RNA-neomycin complex 2.25 |

- E_{NOE} and E_{nhb}: NOE energy and dihedral angle energy resulting from a square well potential function with force constants of E_{NOE} = 50 kcal mol⁻¹ Å⁻² and E_{nhb} = 10 kcal mol⁻¹ Å⁻².
- r.m.s. deviation was calculated for the RNA except the loop region (C29–G36).
Several intermolecular NOEs between neomycin and TAR can be observed in the two-dimensional spectra, but line broadening and low intensity of these signals, which can be attributed to the dynamic properties of the neomycin-TAR complex, do not allow complete assignment of resonances to individual protons. Only two very weak cross-peaks were identified as originating from G44 H-8. In the three-dimensional spectra, all intermolecular NOEs were below the limit of detection. In the

Fig. 6. a, stereoview of the 17 lowest energy structures of the TAR-neomycin complex. TAR RNA is represented by a yellow tube following the phosphate backbone. Neomycin is represented by sticks, and rings have different colors, cyan (I), magenta (II), green (III), and blue (IV). The drug is bound in the minor groove in the lower stem and bulge region of TAR. The stem is well defined, and the apical loop and the bulge show more flexibility. b, stereoview of the eight lowest energy structures of the TAR-neomycin complex. Hydrogen atoms are not shown. Of TAR RNA, only nucleotides 18–28 and 37–44 are shown. Orientation and color coding of neomycin are as in a; U23 is yellow.
In the presence of neomycin, this part of the backbone is bent toward the minor groove, forming a binding pocket for the antibiotic. Arrow 2, in the presence of neomycin, the upper bulge at U 25 is bent toward the minor groove, leaving a cavity into which Tyr 47 may bind. In the presence of neomycin, the upper bulge is displaced toward the major groove occupying the binding cavity for Tyr 47.

The present chemical shift data and, independently, RNase footprinting data (16) indicate that the aminoglycoside binding region stretches from C 19 to G 26 and from U 40 to G 43. Thus, intermolecular NOEs between TAR and ribostamycin were unambiguously assigned to protons of the antibiotic. RNA frequency degeneration in the two-dimensional spectra, however, still left ambiguities.

The lack of a sufficient number of unambiguous distance restraints led us to calculate the structure of the neomycin-TAR complex in two steps. First, we determined the neomycin-bound structure of TAR, omitting the aminoglycoside molecule in the calculations. From two- and three-dimensional NMR spectra of TAR in the presence of neomycin, we derived 365 intramolecular NOE distance restraints to calculate a set of 100 structures of neomycin-bound TAR.

Chemical shift mapping and observation of identical intramolecular NOE patterns in the presence of either neomycin or ribostamycin resulted in the conclusion that neomycin and ribostamycin bind to the same region of TAR. Thus, intramolecular NOEs observed for the ribostamycin complex were considered valid also for neomycin. Given the identity of the first three rings in both aminoglycosides, binding according to the same mechanism is to be expected. Neamine, which is identical to neomycin and ribostamycin in the first two rings, causes changes in the chemical shifts of protons from the same region of TAR as observed for ribostamycin and neomycin, and in other neomycin-RNA complexes contacts responsible for binding specificity are established by atoms from the first two neomycin rings (2, 7). In the tobramycin-RNA complex structures, it is also the 2-deoxystreptamine ring (ring II) that is responsible for specific intermolecular contacts (6).

The present chemical shift data and, independently, RNase footprinting data (16) indicate that the aminoglycoside binding region stretches from C 19 to G 26 and from U 40 to G 43. Thus, intramolecular NOEs were assumed to originate only from protons of nucleotides that are part of or flanking this proposed binding region, while no assumption on minor or major groove binding was made.

Using this strategy, 13 intermolecular distance restraints could be derived from the NMR spectra (Table I) of the RNA-ribostamycin complex, and two ambiguous NOEs observed in the neomycin complex could be assigned unambiguously on the basis of the ribostamycin data. No single strong intermolecular key NOE that a priori allows us to place the aminoglycoside in either the minor or the major groove was identified. NOEs are found between protons of the aminoglycoside and protons of the RNA that are accessible from the minor groove as well as protons accessible from the major groove, a fact that is not surprising, since protons of a ligand bound in the minor groove are expected to be closer than 5 Å to protons in the major groove, and vice versa. However, the combination of the uniquely observed NOEs defines the binding site of the antibiotic. The origin of several pairs of NOEs, for example G 26/H-19 –ring II H-2/H-4 and C 43/U 42 H-9 –ring II H-2/H-4 (Fig. 5), cannot be explained by binding of the antibiotic to the major groove of TAR and thus places the drug in the minor groove.

Further support for minor groove binding is given by the chemical shift data. A binding pocket in the major groove would always include nucleotides of the upper stem for which no significant changes of chemical shift values were observed upon complex formation, rendering major groove binding unlikely.

Structure Calculations Place Neomycin in the Minor Groove of TAR—The NOE-derived intermolecular distances were then used as a starting point for structure calculations of the RNA-neomycin complex; 100 structures of neomycin-bound TAR that resulted from the MD calculations the aminoglycoside was added. Using the RNA-ribostamycin-derived distance restraints, a simulated annealing calculation with subsequent refinement resulted in 17 converged structures with no NOE violations larger than 0.5 Å (Table II).

In none of the calculated models was the aminoglycoside bound in the major groove, reflecting the fact that the set of NOE-derived distance restraints is only compatible with minor groove binding. The structure of neomycin and the stem region of TAR are well defined in the complex. Neomycin is bound in the TAR RNA minor groove, with rings I and II under the triple-U bulge. Ring III is located in the minor groove close to G 21, and ring IV spans the minor groove contacting both A 20...
and G\(^{43}\) (Fig. 6). Stacking of A\(^{22}\) and G\(^{26}\) allows formation of a continuous helix in the RNA stem. A\(^{22}\) and U\(^{40}\) form a Watson-Crick base pair not obvious from the experimental data and therefore not introduced by hydrogen bonding restraints in the calculations. Both bases are propeller-twisted and buckled against each other, explaining the absence of a strong imino proton signal of U\(^{40}\). The minor groove is widened from G\(^{19}\)-G\(^{43}\) to A\(^{22}\)-U\(^{40}\) compared with an ideal A-helix.

The definition of the apical loop and the triple-U bulge is low, reflecting conformational flexibility of these regions (Fig. 6). In all 17 structures, a binding pocket is formed in the deepened minor groove. Bordering on this pocket is the base of G\(^{26}\) that is shifted toward the minor groove relative to A\(^{22}\). Rings I and II of neomycin are enclosed by the protruding phosphate backbone of the bulge and by its bases. Thus, the aminoglycoside is buried in the minor groove, and its accessible surface is reduced by more than 50% compared with the free molecule. The TAR-neomycin complex is thus substantially different from other well-defined RNA-aminoglycoside complexes in which the antibiotic is bound in the major groove of the RNA.

Although the location of the bases of the bulge is not well defined, the backbone of the bulge shows that the conformation differs significantly from all published TAR conformations; in free TAR, the bulge spans a gap of nearly 10 Å between A\(^{22}\) and G\(^{26}\) (25); in the proposed Tat-bound conformation, U\(^{23}\) is in the major groove, T\(^{23}\) is in the minor groove, and C\(^{24}\) is exposed, while the phosphate of C\(^{24}\) protrudes from the backbone (32); in the presence of Ca\(^{2+}\) ions, the bulge adopts yet another conformation, with all three bases stacked above the backbone, which is looped out toward the apical loop, and U\(^{23}\) on the minor groove and U\(^{25}\) on the major groove side (24). None of the published TAR structures shows a cavity in the minor groove formed by the backbone or bases of the bulge. Neomycin thus induces a conformation of the uridine-rich bulge that differs from all known TAR structures.

**How Neomycin Inhibits Tat Binding**—In the presence of neomycin, the volume of the major groove into which Tat binds is reduced. This is caused on the one hand by a change of the bases of the lower stem toward the major groove. Stacking of A\(^{22}\) and G\(^{26}\) is located in the vicinity of G\(^{21}\) and A\(^{22}\) in all structures; the ring II C-1 amino group is close to the phosphate backbone at U\(^{42}\) or O-4 of U\(^{42}\), suggesting that RNA recognition is achieved via the neamine core of the aminoglycosides.

Use of neomycin as an anti-HIV drug would be strongly hampered by its ability to bind to a wide range of RNA structures (1, 8, 9) and to inhibit other biological functions. In all biological systems so far, neomycin is bound deep in the RNA major groove (2, 13, 14), contrasting with the TAR-neomycin complex structure, where part of neomycin ring IV is accessible from the surface (Fig. 6). Thus, additional, bulky groups or even other small molecules attached to ring IV that prevent major groove complexes from being established may be accommodated in this complex, leading to reduction of the toxicity of neomycin while conserving high TAR affinity.

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