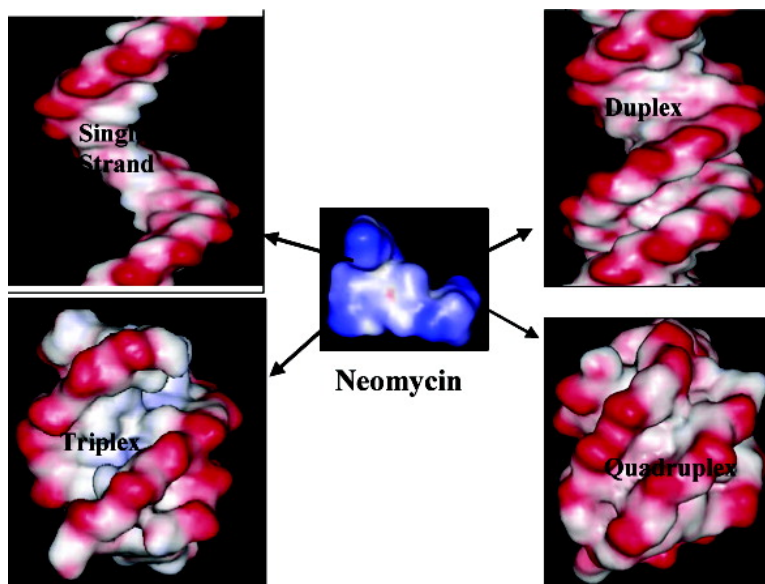## Communication

Aminoglycoside (Neomycin) Preference Is for A-Form Nucleic Acids, Not Just RNA: Results from a Competition Dialysis Study

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Aminoglycoside (Neomycin) Preference Is for A-Form Nucleic Acids, Not Just RNA: Results from a Competition Dialysis Study

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Since the discovery of aminoglycosides by Selman Waksman more than 50 years ago,[1] most attention has focused on their binding to rRNA[2] and recently various other RNA structures.[3] RNA affinity and discrimination by aminoglycosides is modulated by the interplay of nonspecific electrostatic forces, which are critical for affinity, and few specific interactions.[4] The flexible and polycationic nature of the aminoglycoside antibiotics allows them to preferentially bind to prokaryotic ribosomal RNA, but also allows binding to a variety of unrelated RNAs, group I introns, a hammerhead ribozyme, the RRE transcriptional activator region from HIV (which contains the binding site for the Rev protein), the 5′-untranslated region of thymidylate synthase targets for important enzymes such as ribonuclease H and reverse mRNA, a variety of RNA aptamers from in vitro selection, and human mRNAs.[5] Aminoglycoside charge has been suggested to be a necessary evil, leading to increased affinity, at the price of increased promiscuity and inefficient cellular uptake.

Our previous work has shown the remarkable ability of neomycin and other aminoglycosides and conjugates to stabilize DNA, RNA, and hybrid triple helices.[6–10] Neomycin was shown by us to induce the stabilization of DNA·RNA hybrid duplexes as well as hybrid triple helices.[6] This significantly added to the number of nucleic acids (other than RNA) that aminoglycosides have been shown to target. A clear requirement then arose for a quantitative assay to determine the relative binding affinities for host triplex, duplex DNA, single-stranded (DNA/RNA), and other possible nucleic acid targets (tetraplex) for a given aminoglycoside ligand. Fortunately, a rapid technique has now been established by Chaires for this exact purpose, using a thermodynamically rigorous competitive equilibrium dialysis method that exploits therapeutically useful drug concentrations.[11,12] In the assay, solutions of different nucleic acid structures (of identical concentration) are dialyzed simultaneously against a common solution of ligand using appropriately buffered conditions. After equilibration, the amount of ligand bound to each DNA is measured by spectrophotometry. More ligand accumulates in the dialysis tube containing the structural form of highest binding affinity and, because all of the DNA samples are in equilibrium with the same free ligand concentration, the amount of ligand bound is directly proportional to the binding constant for each conformational form. Thus, comparison between the DNA samples gives a rapid and thermodynamically reliable indicator of structural selectivity for any given ligand.

Because aminoglycosides do not have a chromophore for spectrophotometric analysis, we present our findings from competition dialysis of three acridines with increasing positive charge (Figure 1). Competition dialysis studies were carried out using 9-aminoacridine, quinacrine, and a neomycin-acridine (neo-acridine) conjugate[13] against 14 different nucleic acids (Scheme 1). Going from 9-aminoacridine to neomycin-acridine, one can begin to parse the effect of neomycin conjugated to the acridine chromophore.

At first sight, dialysis of neomycin-acridine (Figure 1) shows highly promiscuous binding with little preference for any specific nucleic acid structure, except for a clear preference for RNA.
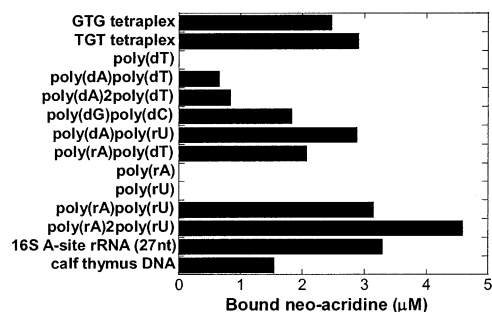

Figure 1. Competition dialysis results of neo-acridine (1 μM) with various nucleic acids; 180 μL of different nucleic acids (75 μM per monomeric unit of each polymer) were dialyzed with 400 mL of 1 μM neomycin-acridine in BPES buffer (6 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1 mM Na_2-EDTA, 185 mM NaCl, pH 7.0) solution for 72 h. Among comparable single-stranded, duplex, and triplex structures, maximum binding is always observed with the triplexes.

Scheme 1. Structures of Neomycin, Aminoacridines, and the Neomycin-acridine Conjugate
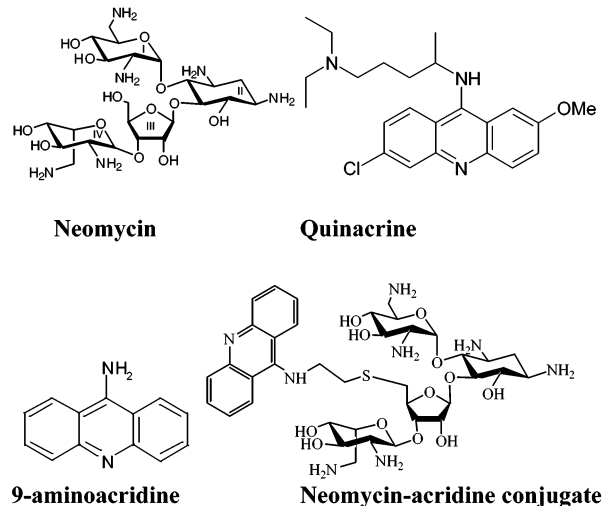
This seemingly promiscuous binding leads to a different picture upon careful analysis of the dialysis data. All three drugs showed comparable binding to one nucleic acid: calf thymus DNA (Supporting Information). Calf thymus DNA also represents a standard duplex DNA. This observation was used to replot the dialysis results to emphasize differences relative to that standard. These results are shown in Figure 2 and better illustrate the change in specificity of the different acridines toward different nucleic acids. While 9-aminoacridine and quinacrine show a clear preference for DNA triplex, neomycin-acridine conjugate binding to RNA triplex is much greater than that to DNA triplex and even better than the natural aminoglycoside RNA target: eubacterial 16S A-site.

Drug binding is also observed with DNA as well as RNA duplex, and even with DNA tetraplex. The binding to DNA tetraplex is
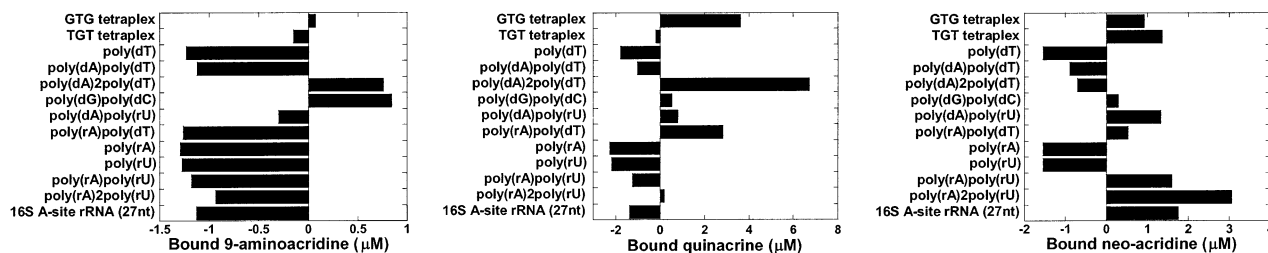
Figure 2. Competition dialysis results (difference plots, with calf thymus DNA as reference) of 9-aminoacridine, quinacrine, and neo-acridine (1 μM) with various nucleic acids. Experimental conditions were identical to those in Figure 1. Maximum binding of neo-acridine is observed with nucleic acids that can adopt the A-type conformation. (Similar results are obtained with 100 nM drug and 7.5 μM nucleic acid concentrations; unpublished results.)

still lower than that to the RNA triplex. RNA·DNA duplexes are even better targets than DNA homoduplexes, poly(dA)·poly(rU) hybrid duplex being comparable in binding to the tetraplexes. Our experiments with aminoglycoside natural products have shown no effect on stability of A·T rich duplex DNA (in the presence of salt), suggesting weaker nonproductive binding. Triplexes are then the targets of choice for neomycin. Neomycin-acridine shows a remarkable binding preference to RNA triplex that has previously not been observed. A big surprise, however, is the significant binding observed with the poly(dG)·poly(dC) duplex.

Neo-acridine binding to RNA triplex was then investigated by UV thermal melts, ITC, and viscometric and CD titrations (Supporting Information). Thermal denaturation in the presence of neo-acridine shows an increase in $T_{m3\rightarrow2}$ at low drug concentrations. At higher drug concentration, duplex is stabilized as well. Neomycin has previously been shown by us to be one of the best stabilizers of an RNA triple helix.[8] Viscosity measurements show a clear groove binding (shortening of RNA triplex length) upon titration of neomycin as well as neo-acridine into the triplex (Supporting Information). ITC titrations show two binding sites for drug (neomycin as well as neo-acridine) binding to the triplex, with K_d in the nanomolar range (approaching the upper limits of analysis by ITC).

What then is the common thread that holds together RNA duplex/triplex, DNA−RNA hybrid duplexes, DNA tetraplexes, and the poly(dG)·poly(dC) duplex? The answer lies in their propensity toward an A-type conformation. RNA duplex structures are known to adopt an A-type conformation, as are the hybrid duplexes.[14] dG·dC rich DNA duplex sequences[15] have also been shown to have a high propensity for the A-form in the presence of cations (including neomycin),[16] and CD studies have shown the A-like conformation of G4 tetraplexes.[17] Further evidence of A-type preference is observed with the change in the CD spectrum of poly(dG)·poly-(dC) upon inclusion of neo-acridine. A shift in λ_{max} from 257 to 267 nm (Supporting Information) and increased signal in this range, in the presence of drug, is strongly indicative of a B→A transformation, as observed by Wang[16] as well as Kypr[17] in similar CD experiments. Additionally, the differences in binding to DNA·RNA hybrids can be attributed to the fact that poly(dA)·poly(rU) has been known to adopt an A-type conformation whereas poly-(rA)·poly(dT) can exist in the B-form.[14]

Electrostatic complementarity has been successfully used to explain the structural basis of RNA binding to their aminoglycoside substrates.[3,4] Perhaps the best complementarity for aminoglycosides with a natural target is observed with eubacterial ribosomal 16S A-site. The structural basis of A-form specificity may be related to the closeness of the two negatively charged sugar−phosphate backbones along the major groove in A-DNA, which can be effectively neutralized by the multivalent positively charged amines

of aminoglycosides. Competition dialysis of a pyrene-neomycin conjugate[18] shows a similar preference for A-form nucleic acids (unpublished results). Groove recognition of triplexes and tetraplexes has been an elusive feat, where such charged polyamine binding factors may be the key to opening this Pandora's box.

To conclude, this work clearly suggests that aminoglycoside specificity (neomycin, in a high nM to low μM range) is for nucleic acid forms that show some features characteristic of an A-type conformation (RNA triplex,[8] DNA−RNA hybrid duplex,[6] RNA duplex,[19] DNA triplex,[7−9,20] A-form DNA duplex,[16] and DNA tetraplex[17]), rather than for naturally occurring RNA. While this work does not question that aminoglycoside's mechanism of drug action involves binding to rRNA, it does challenge, as a matter of biochemical principles, the common belief that aminoglycoside specificity is simply for duplex RNAs.

Supporting Information Available: CD spectra, melting curves for triplex/duplex, ITC/viscometric titration plots (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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