Neomycin-Induced Hybrid Triplex Formation

Dev P. Arya,* R. Lane Coffee, Jr., and I. Charles

Laboratory of Medicinal Chemistry
Department of Chemistry, Clemson University
Clemson, South Carolina 29634

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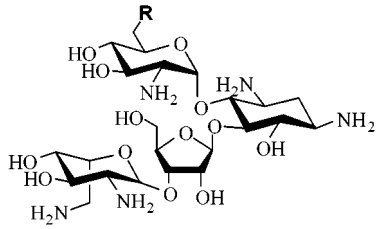
There has been considerable interest in the stability and specificity of recognition in triplexes consisting of both RNA and DNA strands. Biologically important triplexes could be constructed from single stranded RNA and duplex DNA.[1,2] Ribonucleoproteins can act as repressors with mRNA providing the sequence specificity for triple strand formation.[3] RNA·DNA hybrid duplexes are the primary targets for important enzymes such as ribonuclease H and reverse transcriptase.[4,5] Stable RNA·DNA triplexes have been shown to inhibit RNA polymerase,[6] DNAase I and RNAase.[7] Ligand-induced formation of new classes of hybrid triplexes potentially has important implications in the recognition of RNA·DNA hybrid duplexes by cellular and viral proteins.[8]

Telomerase inhibition can be achieved by targeting hTR RNA component of the enzyme via formation of an RNA·DNA hybrid.[9] The polypurine tract (PPT) of HIV and other retroviruses is essential during retroviral replication and is a potential target for triple-helix approaches.[10] The PPT resides in the viral RNA which is transcribed into DNA by the viral reverse transcriptase. The RNA is digested by viral RNAse H except at the polypurine tract where an RNA·DNA hybrid resists hydrolysis and the RNA serves as a primer for the second strand DNA synthesis.[10a,b] Only six of the eight hybrid triplexes are stable under physiological conditions.[11] Triple helix formation is conceivable with the various intermediates: SS RNA, SS DNA, RNA·DNA hybrids, or DS DNA.

Stabilization of Poly(rA)·2Poly(dT) triplex can only be achieved under molar salt conditions.[12] Previous work by Breslauer has shown berenil to be the most effective Poly(rA)· 2Poly(dT) triplex stabilizer.[8] Our recent work has shown the remarkable ability of aminoglycosides (in particular, neomycin, Scheme 1) to stabilize RNA and DNA triple helices.[14] The triplex stabilization effect of neomycin was shown to be the highest among all groove binders previously studied (which tend to prefer the duplex structures). In our quest to expand the triplex stabilization potential of neomycin, we report the remarkable ability of neomycin to induce hybrid DNA·RNA·DNA as well as DNA·RNA·RNA triplexes.

Scheme 1. Structure of Neomycin (R = NH_2) and Paromomycin (R = OH)[a]



[a] For other aminoglycosides, see Supporting Information)

Aminoglycosides are also shown to stabilize the hybrid DNA·RNA duplex.

Without any ligand present, only the duplex transition for Poly(rA)·2Poly(dT) complex, that corresponds to rA·dT melting, is seen (Figure 1). Addition of various aminoglycosides ($r_{db} \leq 1$) was carried out to investigate their effect on inducing the hybrid triplex (Figure 2 and Supporting Information). At lower concentrations of aminoglycosides, the duplex transition shows an increase in $T_{m2\rightarrow1}$ ($r_{db} = 0-0.6$).[13] At a concentration of 20 μM neomycin ($r_{db} = 0.66$), two clear transitions are observed. Further increase in concentration of neomycin does not show much change in triplex/duplex stabilization. Neomycin is the only aminoglycoside that is able to induce triplex formation (UV/CD melts). The melting curves for Poly(rA)·2Poly(dT) in the absence and presence of berenil and neomycin are shown in Figure 1. Our results clearly show that neomycin can induce Poly(rA)·2Poly(dT) triplex formation much more effectively than berenil. At 20 μM drug concentration, neomycin induces the formation of Poly(rA)·2Poly(dT) triplex ($T_{m3\rightarrow2} = 55.0$) much more effectively than berenil ($T_{m3\rightarrow2} = 34.2$). These concentrations of neomycin, however also show considerable precipitation of the complex, leading to a lower hyperchromicity in triplex melting (than that observed with berenil, Figure 1).

We then turned our attention to stabilization of 2Poly(rA)·Poly(dT) triplex. Neomycin is the only aminoglycoside that induces the formation of the triplex, and it does so at much lower concentrations than needed for stabilization of (rA)·2 (dT) triplex (Supporting Information). Surprisingly however, higher concentrations of neomycin lead to three transitions (Figure 3). At low neomycin concentration, the solution contains duplex rA·dT, with unbound excess rA. As neomycin concentrations is increased, the drug can induce the hybridization of excess SS RNA with the duplex, but can also bind to SS RNA that leads to a stable folded structure with poly rA. This was independently confirmed by UV/CD melts of poly rA in the presence of neomycin. At 230, 233 nm, only the triplex/duplex melts are observed (−ve transitions). At 243 nm, only rA and rA·dT melts are observed (−ve transitions). At 263 nm, all three transitions become visible (rA, 2rA·dT +ve; rA·dT −ve). The additional transition at 47 °C was then, upon careful examination of CD and UV melts, assigned to the melting of rA single strands (neomycin and other aminoglycosides are known to bind to RNA).[15] Despite the competition with SS RNA binding, neomycin is able to induce the formation of 2Poly(rA)·Poly(dT) triplex.

To confirm the identity of triplex melting transitions, UV/CD Job plots were carried out (Supporting Information). In the presence of neomycin, a minimum is seen at 66% poly(rA) {2rA·dT}; as well as a minimum at 100% rA (SS RNA). To observe the minimum that corresponds to Poly(rA)·2Poly(dT) triplex, a Job plot of Poly(rA)·Poly(dT) duplex with Poly(dT) at 20 μM neomycin was necessary. As shown in Supporting Information, a minimum is clearly observed at 50% Poly(dT) (260, 280 nm).

* To whom correspondence should be sent. Telephone: 1-864-656-1106. Fax: 1-864-656-6613. E-mail: dparya@clemson.edu.

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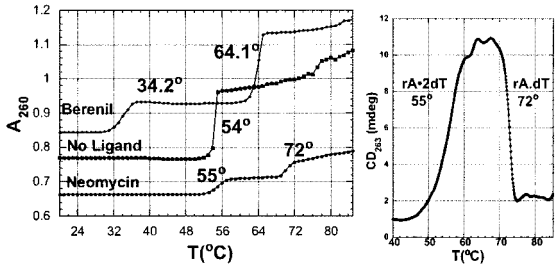

Figure 1. UV Melting curves (left) for Poly(rA)·2Poly(dT) complex showing ligand induced triplex formation in the presence of 20 μM berenil and 20 μM neomycin ($r_{db} = 0.66$); CD Melting curves (right) for Poly-(rA)·2Poly(dT) in the presence of 20 μM neomycin; solution conditions: rA: 30 μM/base triplet; 18 mM NaCl; 10 mM sodium cacodylate; 0.1 mM EDTA, pH 6.8.

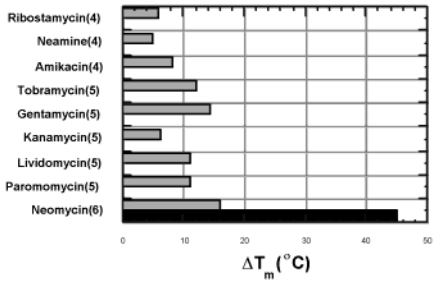

Figure 2. Effect of added aminoglycoside ($r_{db} = 0.66$) on the stabilization of rA·dT duplex (gray) and on inducing rA·2dT triplex (black). Number of amines in each aminoglycoside is shown in parentheses. $\Delta T_{m3\rightarrow2}$ is calculated by assuming a $T_{m3\rightarrow2}$ of 10 °C in the absence of neomycin (no transition seen).

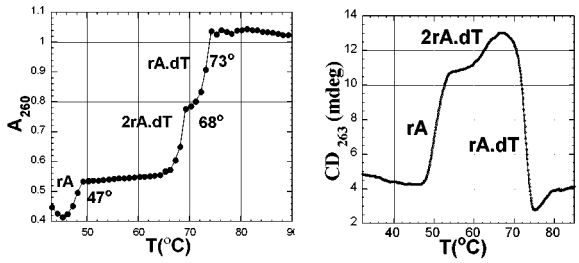

Figure 3. Melting curves (UV left, CD right) for 2Poly(rA)·Poly(dT) complex showing single strand (rA); triplex (2rA·dT) and duplex (rA·dT) melts in the presence of 25 μM neomycin; dT: 30 μM/base triplet; 18 mM NaCl; 10 mM sodium cacodylate, 0.1 mM EDTA pH 6.8.

Most aminoglycosides (4−30 μM, $r_{db} = 0-1.00$) show no effect on stabilizing the triple helix. At higher concentrations ($r_{db} = 0.66-1.00$, Figure 2, also see Supporting Information), most aminoglycosides with five or more amines are able to stabilize the hybrid duplex (increasing $\Delta T_{m2\rightarrow1}$, without inducing a $T_{m3\rightarrow2}$). The difference between the effectiveness of paromomycin and neomycin is indeed remarkable. The structural difference between the two (Scheme 1) is a positively charged amino group (present in neomycin), replacing a neutral hydroxyl group (present in paromomycin). While this leads to a small difference of 4−5 °C in $T_{m2\rightarrow1}$ values (duplex melt at $r_{db} = 0-0.66$), paromomycin is simply unable to induce triplex formation ($T_{m3\rightarrow2}$) under these concentrations. Lividomycin, a paromomycin analogue with a polyhydroxy hexose tether, is equally ineffective in inducing $T_{m3\rightarrow2}$ transition under these conditions (For the change in $\Delta T_{m3\rightarrow2}$ and $\Delta T_{m2\rightarrow1}$ values upon increasing concentration of these three antibiotics, see Supporting Information).

Recent studies have found many RNA molecules that can bind aminoglycosides: group I introns,[15] a hammerhead ribozyme,[16] the RRE transcriptional activator region from HIV, (which contains the binding site for the Rev protein),[17−19] the 5′-untranslated region of thymidylate synthase mRNA,[20] and a variety of RNA aptamers from in vitro selection.[21,22] RNA·DNA hybrids are transiently formed in many vital biological processes, including DNA replication,[23] telomere replication by telomerase,[24] and the replication of HIV (and other retroviruses by reverse transcription).[23] Our finding that aminoglycosides can stabilize DNA·RNA triplexes, hybrid duplexes, and that neomycin can even induce hybrid triplex formation suggests that these higher order structural forms should be taken into account when considering the mechanism of action of these antibiotics in vivo.

While DNA triplex structures have been solved by NMR,[25] structural information on RNA triplexes as well as hybrid triplexes with RNA (polypurine strand−RNA) is not yet available.[26] Our modeling/binding studies with DNA triplexes have suggested that neomycin is unique in targeting the wider Watson−Hoogsteen groove. Surface electrostatics (primary amine in ring I, absent in paromomycin) and shape complementarity of neomycin allow it to target the W−H groove helping bridge the two pyrimidine strands together.[28]

The results of our experiments described here identify a new groove binding ligand (neomycin) selective for inducing triplexes that would normally require molar salt concentrations. Aminoglycosides are also shown to stabilize hybrid duplex structures suggesting that aminoglycoside function may be related to structures other than intramolecular RNA forms (in concert with our previous studies).[14] A recent study has shown the binding of RNA·DNA hybrid duplex to various intercalator complexes.[29] Groove recognition of these hybrid duplexes and triplexes presents us with a new motif of recognition for higher-order RNA/DNA nucleic acid forms. The structural details of these complexes are underway in our labs and will be reported soon.

Supporting Information Available: CD spectra and melting curves (CD/UV) for triplex/duplex denaturation in the presence of different aminoglycosides, T_m values, for poly(rA)·2poly(dT) and 2poly(rA)·poly-(dT) triplex/duplex modeling details for neomycin binding to triplex groove (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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