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Aminoglycoside−Nucleic Acid Interactions: Remarkable Stabilization of DNA and RNA Triple Helices by Neomycin

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Abstract: The stabilization of poly(dA)·2poly(dT) triplex, a 22-base DNA triplex, and poly(rA)·2poly(rU) triple helix by neomycin is reported. The melting temperatures, the association and dissociation kinetic parameters, and activation energies (E_{on} and E_{off}) for the poly(dA)·2poly(dT) triplex in the presence of aminoglycosides and other triplex binding ligands were determined by UV thermal analysis. Our results indicate that: (i) neomycin stabilizes DNA triple helices, and the double helical structures composed of poly(dA)·poly(dT) are virtually unaffected. (ii) Neomycin is the most active and triplex-selective stabilization agent among all aminoglycosides, previously studied minor groove binders, and polycations. Its selectivity ($\Delta T_{m3\rightarrow2}$ vs $\Delta T_{m2\rightarrow1}$) exceeds most intercalating drugs that bind to triple helices. (iii) Neomycin selectively stabilizes $\Delta T_{m3\rightarrow2}$ for a mixed 22-base DNA triplex containing C and T bases in the pyrimidine strand. (iv) The rate constants of formation of triplex (k_{on}) are significantly enhanced upon increasing molar ratios of neomycin, making triplex association rates closer to duplex association rates. (v) E_{on} values become more negative upon increasing concentration of aminoglycosides (paromomycin and neomycin). E_{off} values do not show any change for most aminoglycosides except neomycin. (vi) Aminoglycosides can effectively stabilize RNA {poly(rA)·2poly(rU)} triplex, with neomycin being one of the most active ligands discovered to date (second only to ellipticine). (vii) The stabilization effect of aminoglycosides on triple helices is parallel to their toxic behavior, suggesting a possible role of intramolecular triple helix (H-DNA) stabilization by the aminoglycosides.

Introduction

Triple helix formation recently has been the focus of considerable interest because of possible applications in developing new molecular biology tools as well as therapeutic agents[1−7] and because of the possible relevance of H-DNA structures in biological systems.[1,8] In intermolecular structures, an oligopyrimidine·oligopurine sequence of DNA duplex is bound by a third-strand oligonucleotide in the major groove.[9,10] Specific inhibition of transcription has been shown by means of triplex formation at poly(purine/pyrimidine) sites in promoter sequences [for example, in the promoter of the α subunit of the interleukin-2 receptor to the NF-κB in the c-*myc* promoter,[11−13] and to an Sp1 transcriptional activator site in the Ha-*ras* gene].[14]

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Triplex formation also directly inhibits transcription by blocking RNA polymerase.[15,16] Recently, to monitor endogenous gene modification by triplex-forming oligonucleotides (TFOs) in a yeast model, inactivation of an auxotrophic marker gene has been reported by inserting target sequences of interest into its coding region.[17] Hélène has shown that a phosphoramidate TFO reaches its target sequence, forms cross-links, and generates mutations at the expected site via a triplex-mediated mechanism.[17] Association of a third strand with a duplex, however, is thermodynamically weaker and kinetically slower than duplex formation (eq 1).[18,19]

$$\mathrm{dA{\cdot}dT} + \mathrm{dT} \underset{k_{\mathrm{off}}}{\overset{k_{\mathrm{on}}}{\rightleftharpoons}} \mathrm{dA{\cdot}2dT} \qquad (1)$$

Rates of triple helix formation (second-order rate constants $\sim 10-10^3$ M^{-1} s^{-1})[19−21] are slow (3−4 orders of magnitude) compared to the rate constants of duplex recombination ($\sim 10^6$ M^{-1} s^{-1}).[18,22] Benzopyridoindole derivatives (BePI, for structures, see Supporting Information) were the first molecules reported to strongly stabilize triple helices even though they have a preference for T·A·T stretches.[23−26] Several other intercalators[27−35] as well as various DNA minor groove ligands have also been shown to bind to DNA triple helices. Intercalators usually stabilize to a greater extent triple helices containing T·A·T triplets, whereas minor groove binders usually destabilize triplexes, except in a particular case where the triple helix involved an RNA strand.[6,36] In general, most ligands that stabilize triple helices either intercalate, bind in the minor groove, or carry positively charged functional groups.[37] The intercalating ligands acridine,[37,38] proflavine,[37] fused-ring polycyclic compounds,[39] benzo[*e*]pyridoindole derivatives,[40] ruthenium complexes,[41] ethidium,[42,43] and the alkaloid coralyne[44,45]- (for structures, see Supporting Information) generally tend to be nonspecific in the triplex-to-duplex stabilization, although advances have been made to improve that ratio.[46] Established DNA minor groove-binding ligands distamycin,[47] berenil,[48,49] 4′-6-diamidino-2-phenylindole,[36] netropsin,[50,51] and Hoechst 33258[52] (for structures, see Supporting Information) are also marginally effective stabilizers for nucleic acid triplexes when at least one strand is a ribooligonucleotide. Recently, 3,3′-diethyloxadicarbocyanine (DODC) has been shown to selectively stabilize DNA triple helical structures,[53] although its mode of binding is not yet known. Polycations such as diamines and polyamines,[54−60] bisguanidines,[61] some basic oligopeptides,[62] and comb-type polycations[63] have long been known to stabilize triple helical structures. In our quest for new ligands for triple helix stabilization, we have investigated aminoglycoside anti-

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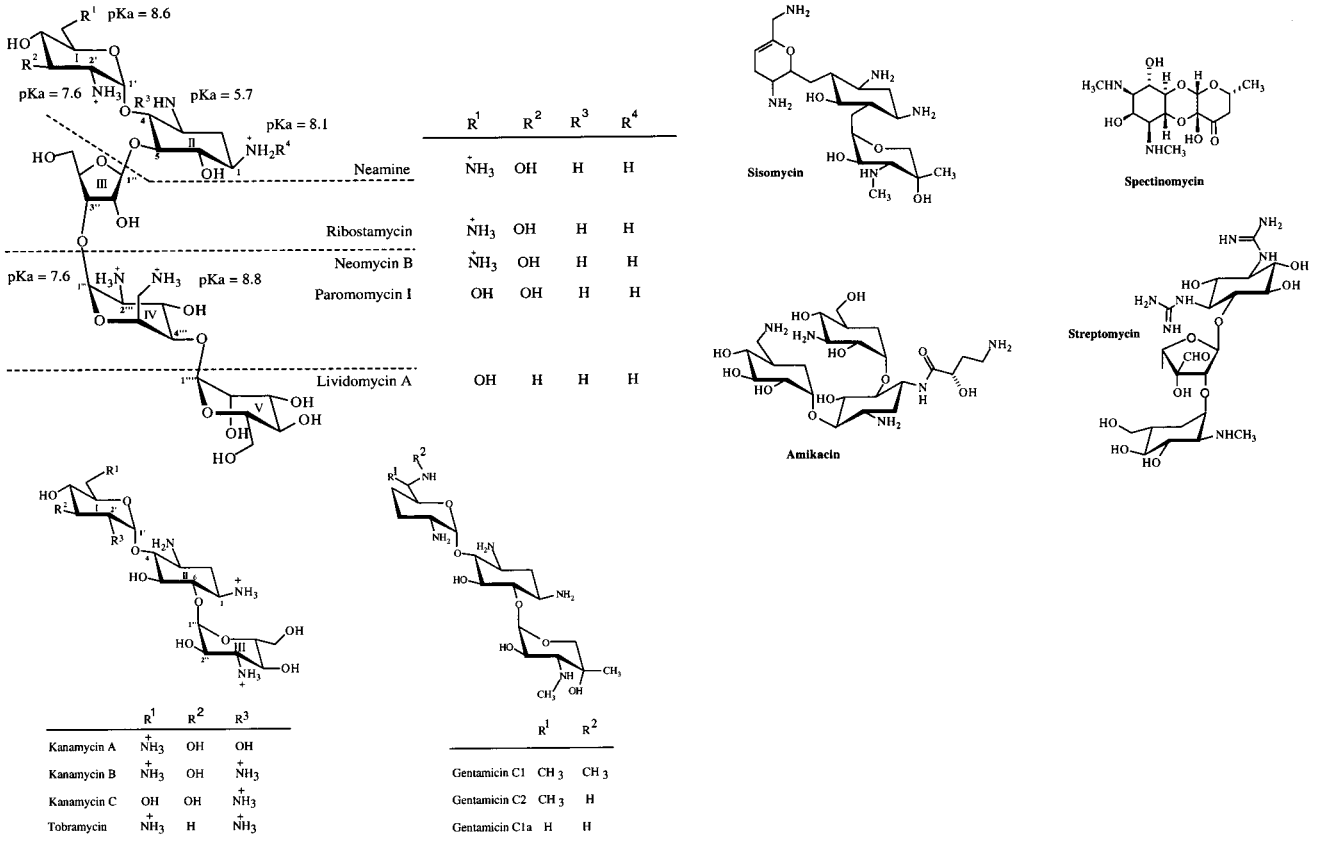
Scheme 1. Structures of Aminoglycosides Used in the Study[a]



[a] Amine pK_a's correspond to those in neomycin[84]

biotics (Scheme 1).[64] Recent work by Rando,[65−72] Tor,[73−75] Wong,[76−80] and other groups[70,81−84] has shown that the binding of aminoglycosides is favored at domains in RNA that are nonduplex in nature. A possible explanation given was the narrow minor groove of duplex RNA that does not allow for aminoglycoside access.[71] Wong has recently shown that 1-amino-3-propanols can bind to phosphodiesters with better affinity than guanidinium groups.[85] Recent studies have also found many RNA molecules that can bind aminoglycosides: group I introns,[86] a hammerhead ribozyme,[74] the RRE transcriptional activator region from HIV[66,72,76] (which contains the binding site for the Rev protein), the 5′-untranslated region of thymidylate synthase mRNA,[70] and a variety of RNA aptamers from in vitro selection.[68,71] A recent report by Pilch describes the binding of some aminoglycosides to RNA double helices.[87] We have recently reported the stabilization of the poly(dA)·2poly(dT) triple helix by neomycin.[64] Our results have shown that neomycin is the most active of all aminoglycosides in stabilizing triple helices and that it does not influence the double helical DNA structures, even at high concentrations. Herein, we report the stabilization of DNA as well as RNA triple helices by aminoglycosides. The kinetics of association and dissociation of DNA triple helix in the presence of aminoglycosides are also presented. Neomycin is also found to significantly enhance and stabilize triplex formation of a short (22-base) DNA sequence in the pyrimidine motif. Neomycin and other aminoglycosides are shown to stabilize RNA triple helices at very low concentrations. The toxicity values of neomycin and other aminoglyco-

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sides (LD_{50}) correlate well with their corresponding stabilization effects on DNA triple helices.

Methods

For a description of materials, see Supporting Information. UV spectra were recorded at $\lambda = 220-280$ nm on a Cary 1E UV/vis spectrophotometer equipped with temperature programming. Spectrophotometer stability and λ alignment were checked prior to initiation of each melting point experiment. For the T_m determinations, derivatives were used. Data were recorded every 1.0°. In all poly(dA)·2poly(dT) experiments, the samples were heated from 25 to 95 °C at 5 deg/min, the annealing (95−10 °C) and the melting (10−95 °C) were conducted at 0.2 deg/min, and the samples were brought back to 25 °C at a rate of 5 deg/min. In case of higher concentrations of neomycin (>5 μM) in poly(dA)·2poly(dT) solutions, the heating and cooling curves were run from 30 to 95 °C to avoid precipitation at lower temperatures. This precipitation effect of neomycin is similar to previously studied polycations (spermine and cationic polypeptides).[57,63] For the DNA 22-mer experiments, samples were heated from 25 to 85 °C at 5 deg/min, the annealing (85−5 °C) and the melting (5−85 °C) were conducted at 0.2 deg/min, and the samples were brought back to 5 °C at a rate of 5 deg/min. DNA polymers were dissolved in 10 mM sodium cacodylate buffer (pH 7.2) containing 0.5 mM EDTA. DNA 22-mers were dissolved in 10 mM sodium cacodylate buffer (pH 6.8) containing 0.1 mM EDTA. RNA solutions were dissolved in 10 mM sodium cacodylate buffer (pH 6.8) containing 0.1 mM EDTA. For all experiments, RNA concentrations were 20 μM/base triplet, DNA concentrations were 15 μM/base triplet, and 22-mer concentrations were 1.0 μM/strand. The RNA samples were incubated for 16 h at 4 °C before the melting experiments, which were recorded from 10 to 95 °C at a rate of 0.2 deg/min. There was little precipitation observed with RNA triplex. $T_{m3\rightarrow2}$ was obtained at 280 nm, and $T_{m2\rightarrow1}$ was obtained at 260 nm for RNA melting experiments. RNA melting experiments were also run at 283.5 and 287.0 nm to differentiate between the transitions obtained. Solutions containing poly(dA)·2poly(dT) were prepared by mixing poly(dA) and poly(dT) in a 1:2 molar ratio. The ionic strength, μ, was adjusted with KCl (150 mM) for DNA polymers, and the 22-mer and NaCl (35 mM) was used for RNA polymers. All stock solutions were kept at 4 °C between experiments. In the isothermal kinetic experiments involving the DNA 22-mers (10.5 μM/strand), both duplex (dY·dR) and TFO (dT) solutions were monitored for stable UV absorbance (260 nm) at 8 °C (with triplex being the favored form at these low temperatures) before mixing occurred. Curve fitting was performed with the software supplied by Cary 1E UV/Vis Kinetics Program. Rate constants reported are averages of three or more experiments.

Results and Discussion

(1) Continuous Variation and Thermal Denaturation Studies with Poly(dA)·2Poly(dT) and a 22-Base DNA Triplex in the Presence of Neomycin. To investigate the interaction of neomycin with poly(dA)·2poly(dT) in the presence of 150 mM KCl, we constructed UV continuous variation plots at different wavelengths and temperatures. Continuous variation experiments were carried out with the measurement of complete spectra of each of the different mixtures (Supporting Information). Mixtures of neomycin with poly(dA) and poly(dT) at 10 °C (Figure 1) show breaks at a mole fraction of ~0.66 poly(dT) to 0.34 poly(dA) as well as of ~0.5 poly(dT) to 0.5 poly(dA). These numbers indicate that triple-stranded and double-stranded complexes are formed containing poly(dA)·2poly(dT) and poly(dA)·poly(dT), respectively, in the presence of 0−10 μM neomycin. As neomycin concentration is increased from 2 to 10 μM (Figure 1), there is a lowering of absorbance for triplex, such that the breaks at 0.5 poly(dT) and 0.66 poly(dT) get closer to each other, indicating stabilization of the triplex. At 60 °C the plots (data not shown) have a sharp minima at 0.5 mol % of dT. This confirms the presence of poly(dA)·poly(dT) duplex above 60 °C.

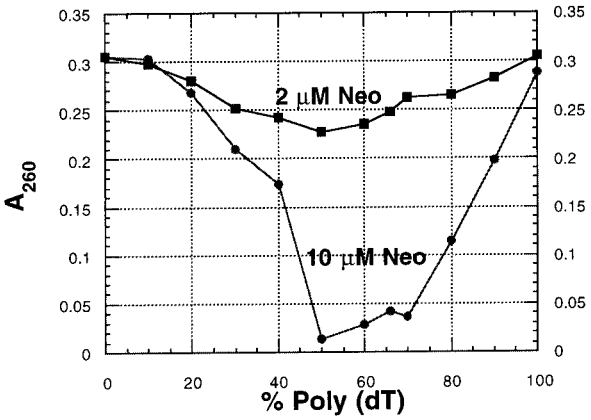

Figure 1. Job plot of poly(dT) (3.73×10^{-5} M) and poly(dA) (3.73×10^{-5}M) at 10 °C in the presence of 2 μM (■) and 10 μM (●) neomycin showing breaks at 50% poly (dT) and 66% poly (dT).

Table 1. UV Melting Temperatures at 260 nm with Increasing Aminoglycoside Concentration in the Presence of 150 mM KCl[a]

	4 μM $r_{db} = 0.26$		25 μM, $r_{db} = 1.67$	
antibiotic	$\Delta T_{m3\rightarrow2}$	$\Delta T_{m2\rightarrow1}$	$\Delta T_{m3\rightarrow2}$	$\Delta T_{m2\rightarrow1}$
neomycin(6)	5.7	1.0	24.7	1.0
paromomycin(5)	2.2	1.1	8.5	0.1
lividomycin(5)	2.1	0.0	3.0	3.0
kanamycin(5,4)	−2.3	0.0	3.0	1.1
gentamycin(5)	2.2	0.0	7.1	0.1
sisomicin(5)	0.8	0.0	11.0	0.1
tobramycin(5)	0.1	0.0	7.1	2.1
amikacin(4)	0.5	0.0	3.3	0.1
neamine(4)	−1.0	0.0	1.1	1.1
ribostamycin(4)	−2.3	0.0	1.8	1.0
streptomycin(3)	−0.8	1.0	−0.9	0.1
spectinomycin (2)	−1.0	0.0	2.2	1.1

[a] Melting transitions of the triplex poly(dA)·2poly(dT) and duplex poly(dA)·poly(dT) are noted as $T_{m3\rightarrow2}$ and $T_{m2\rightarrow1}$, respectively. Without any antibiotics present, the melting temperature of the triplex was 34.0 °C and that of the duplex was 71.0 °C. Number of amines in each compound is indicated in parentheses.

Melting studies of triplexes formed from poly(dA)·poly(dT) and poly(dT) were carried out using UV spectroscopy at 260 and 284 nm. The ratio between poly(dA)·poly(dT) and poly(dT) was 1:1. In the thermal denaturation analysis of poly(dA)·2poly(dT) bound to neomycin, plots of absorbance at 260 and 284 nm (A_{260}, A_{284}) versus temperature exhibit two distinct inflections {$T_{m3\rightarrow2}$ (triplex melting point) = 34 °C and $T_{m2\rightarrow1}$ (duplex melting point) = 71 °C, $\mu = 0.15$}. Triplex stabilization was found to be dependent on neomycin concentration. Table 1 shows that by increasing the molar ratios of neomycin from 0 to 25 μM, r_{db} {ratio-drug(neomycin)/base triplet} = 0−1.67, the triplex melting point is increased by close to 25 °C, whereas the duplex is virtually unaffected. It is remarkable that under these conditions neomycin has little or no effect on the duplex DNA melting (Figure 2). A plot of this change in $T_{m3\rightarrow2}$ and $T_{m2\rightarrow1}$ versus r_{db} (Figure 2) shows that while $\Delta T_{m3\rightarrow2}$ increases upon increasing concentrations of neomycin, $\Delta T_{m2\rightarrow1}$ does not change. This triplex stabilization behavior of neomycin was independently confirmed by using double helical poly(dA)·poly(dT). In the absence of KCl, only one transition is seen for a 1:2 mixture of poly(dA) and poly(dT), which corresponds to the melting of the duplex.[56] Addition of 4 μM neomycin in the absence of any salt leads to two clear transitions (Figure 3), driving the equilibrium in eq 1 to the right.

While neomycin shows a profound effect on stabilizing the triplex of poly(dA)·2poly(dT), it was of interest to investigate

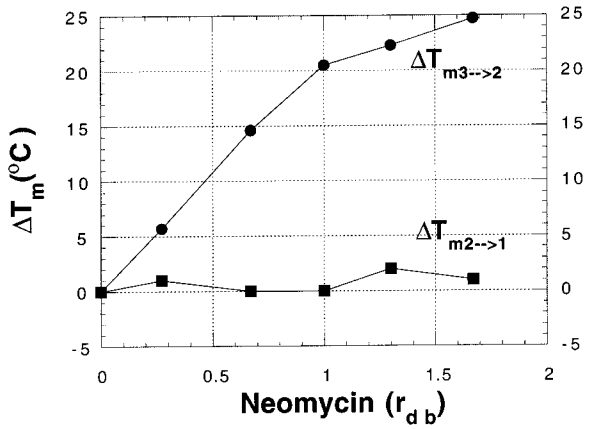

Figure 2. Plots of variation of $T_{m3\rightarrow2}$ and $T_{m2\rightarrow1}$ of poly(dA)·2poly(dT) as a function of increasing neomycin concentration (r_{db} = drug(neomycin)/base triplet ratio). [DNA] = 15 μM base triplet.

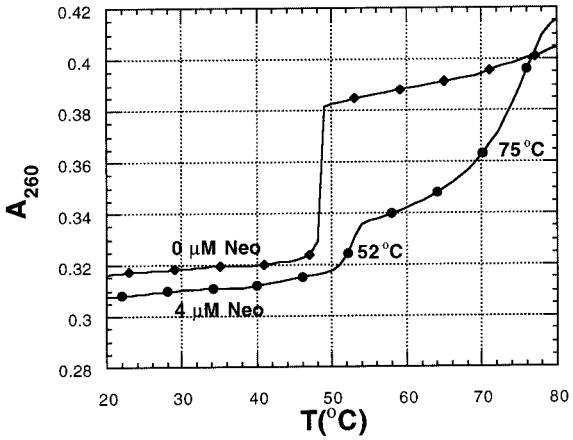

Figure 3. UV melting profile at 260 nm in the absence of KCl at a rate of 0.5 °C/min. 0 μM neomycin (◆) showing duplex melt {poly(dA)·poly(dT)} and 4 μM neomycin (●) showing triplex melt {poly(dA)·2poly(dT)} and duplex melt {poly(dA)·poly(dT)}, respectively. Samples were allowed to incubate at 4 °C for 16 h prior to run. [DNA] = 15 μM base triplet.

its effect on shorter, mixed-base sequences. We carried out thermal denaturation studies of a 22-mer triplex[19] (below) in the presence of neomycin. We found that neomycin stabilizes the triplex of shorter, mixed base (cytosine-containing) sequences, without any effect on the duplex at a pH of 6.8 (Figure 4). At concentrations of up to 22 μM neomycin (r_{db} = 1),

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22dR:    5'd(AAAGGAGGAGAAGAAGAAAAAA)3'
22dY:    3'd(TTTCCTCCTCTTCTTCTTTTTT)5'
22dT:    5'd(TTTCCTCCTCTTCTTCTTTTTT)3'
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$\Delta T_{m3\rightarrow2}$ = +16 °C for the 22dY·dR·dT triplex, while $\Delta T_{m2\rightarrow1}$ = 0 for duplex dY·dR (Figure 4). At higher concentrations, the triplex and duplex transitions merge. These results suggest that neomycin can stabilize DNA triplexes containing a mixed pyrimidine sequence that include cytosine bases.

(2) Thermal Denaturation Studies with Poly(dA)·2Poly(dT) in the Presence of Other Aminoglycosides and Diamines. We then carried out thermal analysis of poly(dA)·2poly(dT) in the presence of other aminoglycosides (Scheme 1, Table 1). Table 1 shows the results of thermal analysis of these experiments. Most aminoglycosides (4−10 μM, r_{db} = 0−0.66) have either no effect or slightly destabilize the triple

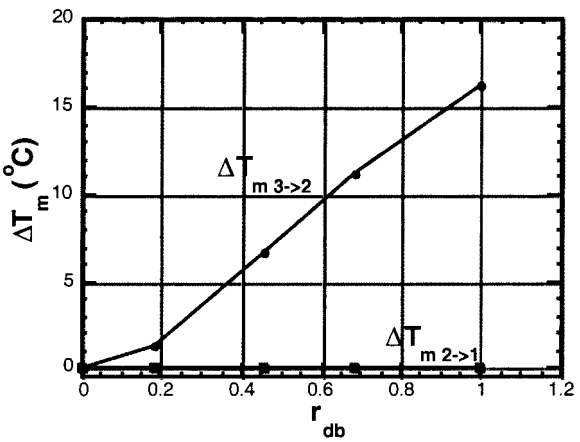

Figure 4. Plots of variation of $T_{m3\rightarrow2}$ and $T_{m2\rightarrow1}$ of the 22-mer dY·dR·dT triplex as a function of increasing neomycin concentration (r_{db} = drug(neomycin)/base triplet ratio).

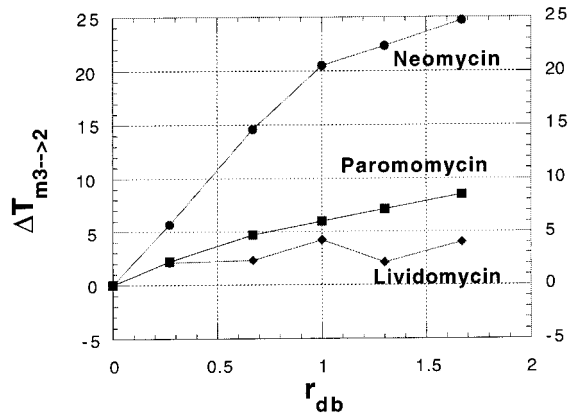

Figure 5. Plots of variation of $T_{m3\rightarrow2}$ as a function of increasing neomycin, paromomycin, and lividomycin concentration (r_{db} = drug/base triplet ratio) on poly(dA)·2poly(dT) triplex.

helix. Sisomicin, paromomycin, and lividomycin are the only other antibiotics that have a stabilizing effect at these low concentrations. At higher concentrations (r_{db} = 0.66−1.67), most aminoglycosides with five or more amines are able to stabilize the triple helix (increasing $\Delta T_{m3\rightarrow2,}$ without significantly affecting the $\Delta T_{m2\rightarrow1}$values). The difference between the effectiveness of paromomycin and neomycin is quite remarkable. The structural difference between the two is a positively charged amino group (present in neomycin), replacing a neutral hydroxyl (present in paromomycin). This leads to a difference of 10 °C in $T_{m3\rightarrow2}$ values (r_{db} = 0.66) and a difference of 16 °C at r_{db} =1.67. At lower concentration of antibiotics (r_{db} = 0.26), paromomycin has little effect on the stability of the triplex. Lividomycin, a paromomycin analog with a polyhydroxy hexose tether, is slightly less effective than paromomycin in increasing $T_{m3\rightarrow2}$ values under these conditions. Table 1, Figure 5 show the change in $\Delta T_{m3\rightarrow2}$ values upon increasing concentration of these three antibiotics. As clearly seen from Figure 5, neomycin is far more effective than paromomycin or lividomycin in stabilizing triple helices ($\Delta\Delta T_{m3\rightarrow2}$ = 20 between neomycin and lividomycin at r_{db} = 1.67).

Neamine (structural subset of neomycin: Scheme 1, Table 1, 4 amino groups), the diamines, pentaethylenehexamine, and aminopropanol (Table 2) have little or no effect on the stabilization of the duplex or the triplex. All aminoglycosides with five amino groups do not show similar stabilization properties. Paromomycin and sisomicin are better stabilizers at high r_{db} values, compared to lividomycin, kanamycin, tobra-

Table 2. UV Melting Temperatures at 260 nm in the Presence of Different Amines (4, 10 μM) and 150 mM KCl

	4 μM amine		10 μM amine	
poly(dA)·2poly(dT) + amine	$\Delta T_{m3\rightarrow2}$	$\Delta T_{m2\rightarrow1}$	$\Delta T_{m3\rightarrow2}$	$\Delta T_{m2\rightarrow1}$
1-amino-3-propanol	1.0	0.0	−1.4	1.0
1,3-diaminopropane	−0.7	−1.0	−0.5	0.1
1,4-diaminobutane	−0.4	0.0	0.0	0.1
1,5-diaminopentane	−2.1	−1.0	−2.4	0.1
spermine	5.7	1.0	7.4	0.0
spermidine	0.5	1.0	−2.0	0.1
pentaethylenehexamine	−0.4	0.1	2.0	1.0

mycin and gentamycin (Table 1). Thus, the polycationic nature of neomycin and other aminoglycosides is not the only explanation for their role in triple helical stabilization. The placement of positive charges in some of these constrained aminoglycosides makes them more suitable for triplex binding than the flexible conformation of other aminoglycosides and di- and polyamines. Geometrical parameters, for example, the distance between the charges, must also play a significant role. The presence of hydroxyl groups and perhaps more importantly their alignment with respect to the amino groups could be responsible for this difference in $T_{m3\rightarrow2}$ values. Rando has previously shown that simple amino alcohols can act as comparative surrogates to aminoglycosides in their RNA-binding activity.[69]

The protonated amino groups in these aminoglycoside antibiotics can serve, in addition to specific hydrogen bonds and van der Waals contacts, as donor groups having complementary electrostatic interactions with the electronegative density created by the fold of the polyanionic triple helix backbone. In addition, the hydroxyl groups, because of their water-like behavior, can replace water molecules and form hydrogen bonds with anionic phosphate oxygens and the heterocyclic atoms of the bases. Since aminoglycosides are highly functionalized polycationic oligosaccharides, interactions between their polar residues (amino and hydroxyl groups) and the DNA backbone and heterocyclic bases are likely to occur. Since most of the amino groups are predominantly protonated at pH 7.0, the overall charge density presented by the aminoglycosides toward the RNA host has been shown previously to be most likely for RNA binding and should be a significant contributor here.[75] Table 1 lists these aminoglycosides in a decreasing number of amino groups present (number of amino groups indicated in parentheses). The pK_a values for the amino groups in neomycin are shown in Scheme 1.[84] Since the pK_a of one of the amino groups is close to 5.6, this is the only amine that remains substantially deprotonated at physiological pH. The number of charges that contribute to triplex stabilization would be limited to five in neomycin, four in paromomycin, lividomycin, and the gentamycin/kanamycin families, since most of these aminoglycosides have one amino group with a substantially lower pK_a. This behavior has been recently studied in the interaction of these aminoglycosides to double helical RNA.[87] Previous work on structure−activity relationships for natural aminoglycosides has also shown that aminoglycosides containing four amino groups show very little ability to bind RNA, whereas the most active derivatives contain five or six amino groups.[75] The difference in selectivity between paromomycin and neomycin (five vs six amines, four vs five positive charges at pH 7) further supports this rather general view of aminoglycoside−nucleic acid interaction.

(3) Stabilization of DNA Triple Helix Poly(dA)·2Poly(dT) by Other Ligands. To assess how neomycin compares to other ligands in stabilizing triplexes, we carried out thermal denaturation analyses of poly(dA)·2poly(dT) triplex in the presence of previously studied intercalators and minor groove binders (Schemes S1 and S2, Supporting Information). The results are shown in Figure 6 and indicate that neomycin is much more active than the minor groove binders (berenil, spermine, Hoechst 33258, Hoechst 33342). The intercalating ligands are equally or more effective at lower concentrations (4 μM) in stabilizing the triple helix (Supporting Information). However, at higher concentrations, the intercalating ligands begin to stabilize the duplex as well, which makes the selectivity of neomycin and the aminoglycosides even more remarkable. The minor groove binders previously studied have little preference for triple helix (berenil, distamycin, and Hoechst dyes). Most groove binders stabilize the duplex much more effectively. A few even destabilize the triplex. Neomycin does not affect the DNA duplex even at concentrations higher than that shown in Figure 6 (150 mM KCl). A host of DNA triplex intercalators (selective and nonselective, at last count close to 300) have been used to stabilize triplex structures, but the selective targeting of DNA/RNA triplex grooves has not been accomplished. There is little information available for antibiotics that bind DNA triplex grooves or RNA triplex grooves. Our work is focused on narrowing this disparity between groove recognition of duplex versus triplex nucleic acids. We present neomycin as one of the first examples that bridge this gap and may thus lead to a novel understanding of the recognition principle(s) involved in selective targeting of triplex grooves. Our work suggests that neomycin is unique in targeting triplex grooves and not duplex grooves, a critically different and important property when compared to other known groove binders which overwhelmingly prefer the W−C duplex minor groove.

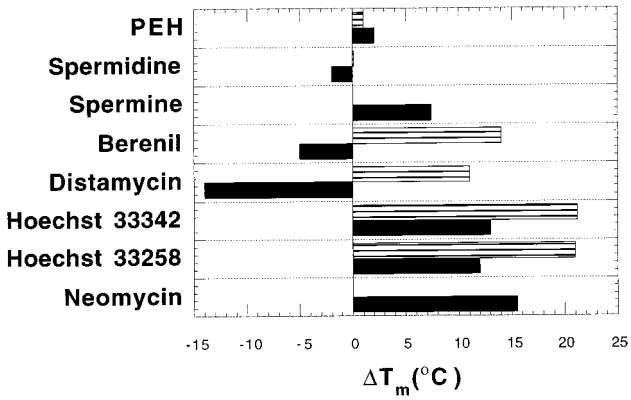

Figure 6. Effect of 10 μM (r_{db} = 0.66) groove binders on the DNA triplex melt{poly(dA)·2poly(dT)} (solid bar) and the duplex melt{poly(dA)·poly(dT)}(striped bar). Distamycin does not show $T_{m3\rightarrow2}$ transition (<20 °C). PEH = pentaethylenehexamine.

(4) Kinetics of Association and Dissociation of DNA Triplexes in the Presence of Neomycin: (a) Poly(dA)·2Poly(dT). During the first transition, annealing and melting curves of complexes formed from poly(dA)·2poly(dT) with neomycin exhibit hysteresis at the rate of heating−cooling employed (0.5 and 0.2 deg/min). Thermal analysis at 284 nm only show hyperchromicity for the transition of the triple helix to the double helix. These measurements confirm that the first transition ($T_{m3\rightarrow2}$) is the destabilization of the triple helix, since (a) rates of formation of triple helices are considerably lower than double helical complexes and (b) triple helical transitions show hyperchromic effect at ∼284 nm.

The hysteresis curves generated (20−60 °C, 0.2 deg/min, Supporting Information) were used to calculate the rates of association and dissociation, k_{on} and k_{off}.[19] As evident from Table

Table 3. Rate Constants of Triplex Association (k_{on}) and Dissociation (k_{off}) and Free Energy of Formation of Poly(dA)·2Poly(dT) Triplex (15 μM /base, 0.15 M KCl) in the Presence of Neomycin at 37 °C (Margin of Error: $k_{on} = \pm 10\%$; k_{off} $\pm 10\%$; $\Delta G = \pm 10\%$, $T_m = \pm 1$°C)

neomycin, μM	k_{on} (M^{-1} s^1)	k_{off}*10^3 (s^{-1})	K_{eq}	ΔG kcal/mol
0	2.65	2.4	0.02	2.4
1	4.05	0.94	0.06	1.7
2	7.20	1.04	0.10	1.4
4	61.5	0.32	2.8	−0.6
10	44.0×10^2	0.77	85.7	−2.7

3, increasing the concentration of neomycin from 0 to 10 μM increases the second-order rate constant (k_{on}) from 2.65 to 4.4 $\times$ 10^3, an increase of more than 3 orders of magnitude at 37 °C, under physiologically relevant salt conditions. The free energies of triplex formation are positive initially (0−2 μM neomycin) since duplex is the favored complex above the triplex melting point (37 °C). A further increase in neomycin concentration shifts the equilibrium to the triplex ($\Delta\Delta G_{0-10\mu M} = 5.13$ kcal/mol), mainly by increasing the k_{on} values. While the k_{off} values do decrease upon increasing the neomycin concentrations (0−10 μM), the effects are minimal at 37 °C (3-fold). We believe this is an important property for a TFO that binds to a target with high fidelity and large association rates. The association rates should be fast enough to compete with protein binding (transcription factors), yet the dissociation rates should be fast enough to only allow binding with high fidelity to the complementary duplex. Ligands that can achieve these criteria in the stabilization of triple helices would tend to be the most successful therapeutically. The 40,000-fold increase of the equilibrium association constant of a DNA triplex, in going from 0 to 40 mM $MgCl_2$, has been shown previously to be due to an increased k_{on} value.[19] The 4200-fold increase in the equilibrium association constant observed here is due to the addition of only 10 μM neomycin (Table 3). Similarly, 300 mM NaCl was shown to lead to a 100-fold increase in k_{on} (at 15 °C).[19] Addition of only 10 μM neomycin leads to a 1600-fold increase in k_{on} (at 37 °C). These comparisons are purely qualitative since previous studies were done on a 22-base pair triplex at 15 °C, in the presence of NaCl.[19] Other studies have evaluated the kinetics of triplex association in temperatures from 15 to 37 °C.[19,21] The rate constants derived here can be extrapolated to 25 °C with a high degree of confidence. However, at temperatures 15−20 °C lower than the melting point of the complex, association rate constants do not always vary linearly at lower temperatures.[88] Duplex recombination rate constants actually show a decrease at low temperatures and almost never exceed the diffusion limit.[88] Further evaluation of the rate constants at lower temperatures will be done using stop-flow kinetics and reported soon.

(4) (b) 22-mer DNA Triplex. Isothermal absorbance decay curves of 22dY·dR·dT[19] triplex (described above) were obtained in efforts to study neomycin's effect on the kinetics of triplex formation of shorter, mixed sequences (Figure 7). In this study, dT (TFO) was combined with varying concentrations of neomycin, and added to duplex dY·dR. Both duplex and TFO were monitored for stable UV absorbance before combining (Figure 7; TFO not shown). A small range of neomycin concentrations was available for study, however, as precipitation occurred at neomycin concentrations greater than 2.0 μM. There is a substantial decrease in $t_{1/2}$ as neomycin concentration increases (Table 4). At higher temperatures (20−30 °C), significantly larger increases in k_{on} are observed, and a complete kinetic analysis will be reported elsewhere. These results suggest that neomycin's role in DNA triplex stabilization is not limited to DNA homopolymers, where bulged or partially looped structures could possibly be responsible for neomycin's observed effect on triplex formation.

Increased salt (KCl and $MgCl_2$) as well as polyamine concentration has been shown to enhance the association rates of triplex formation.[9,10,19,20] Preassociation of neomycin with DNA single strands during the rapid equilibration of the first few base triplets in the triple helix formation may help explain its effect on the increased k_{on} values. This is further supported by our observation that DNA duplex is not stabilized by neomycin as well as by the fact that aminoglycosides are well-known for binding single-stranded RNA structures.[86] Increased concentration of neomycin drives the equilibrium toward triplex formation even in the absence of any salt (Figure 3). Thus, association of neomycin to single-stranded DNA, and not duplex DNA, is perhaps responsible for increased k_{on} values. Similar effects of a single-stranded structure near room temperature have been shown by Breslauer to significantly reduce the enthalpic driving force predicted for duplex formation from nearest-neighbor data, since such data generally are derived from measurements in which the single strands are in their random-coil states.[89] Consequently, as pointed out by Breslauer, "Potential contributions from single-stranded structure must be recognized and accounted for when designing hybridization experiments and when using isothermal titration or batch mixing techniques to study the formation of duplexes and higher-order DNA structures (e.g., triplexes, tetraplexes, etc.) from their component single strands."[89] Ligands that stabilize or destabilize such higher-order structures can do so by contributing to such single-strand structures, and we suggest that to be one of the factors here.

(5) Kinetics of Association in the Presence of Paromomycin and Other Amines: Difference a Charge Makes. Paromomycin and neomycin differ structurally by one amino group (present in neomycin). This leads to a difference of 10 °C in the $T_{m3\rightarrow 2}$ values and a large difference in k_{on} values (400 times), whereas the k_{off} values of the two antibiotics differ by less than a factor of 2 (Table 1, Table 5).

The polyamines spermine and spermidine, which have been widely used for triplex stabilization, show widely different stabilization under the conditions of our assay (Table 2), as previously reported.[54,57−59] While spermine shows an increase of 5 °C in $T_{m3\rightarrow 2}$ value, spermidine is simply ineffective at these low concentrations (Table 2). Spermine stabilizes the triple helix by increasing the k_{on} values (140.0 M^{-1} s^{-1}, Table 5), which is 30 times less than the neomycin stabilization ($k_{on} = 4.4 \times 10^3$ M^{-1} s^{-1}, Table 3).

Spermine, however, does lower the k_{off} values more than neomycin (0.2×10^{-3} s^{-1} compared to 0.77×10^{-3} s^{-1} in the presence of neomycin). Cationic peptides have been previously shown to stabilize triplexes with an ability similar to that of spermine.[62] Neomycin clearly is better than spermine in increasing the k_{on} values as well as in increasing the $T_{m3\rightarrow 2}$ values. The rate constants of association and dissociation for the triplex in the presence of diamines and 1-amino-3-propanol (Supporting Information) under similar conditions show little variation.

(6) Activation Energies and Mechanism of Helix Formation. The negative values for E_{on} (activation energy for k_{on}) are

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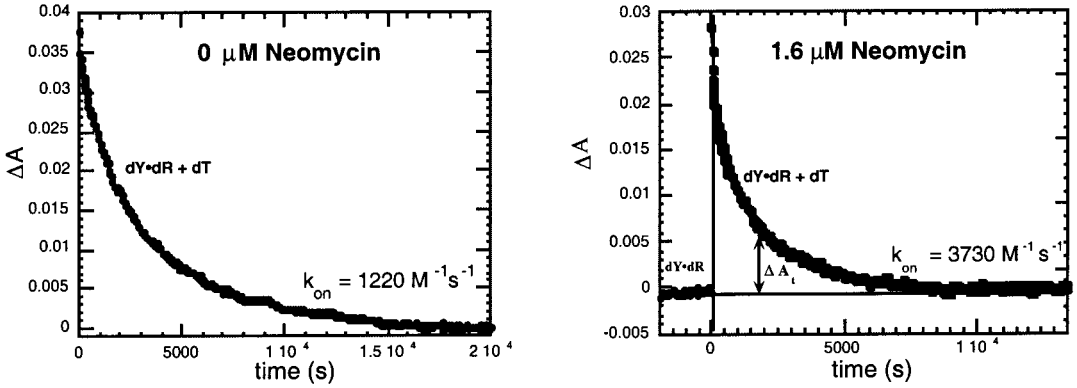

Figure 7. Rate enhancement of 22dY·dR·dT triplex formation by neomycin. Absorbance decay curves for 22dY·dR·dT formation in the presence of 0 (left) and 1.6 μM (right) neomycin. Both duplex and TFO were mixed in an equimolar ratio. Duplex dY·dR (also shown) was monitored for stable absorbance before mixing. Conditions: 150 mM KCl in 10 mM sodium cacodylate, 0.1 mM EDTA buffer, pH 6.8. [22mer] = 0.5 μM/strand; λ = 260 nm; T = 8° C.

Table 4. Second Order Rate Constants and Corresponding Half-Lives for Formation of 22dY·dR·dT Triplex in the Presence and Absence of Neomycin ([22mer] = 0.5 μM/strand; μ = 0.15; pH = 6.8; T = 8 °C)

neomycin, μM	k_{on} (M^{-1} s^{-1})	$t_{1/2}$ (s)
0	1220 ± 30	1640
0.4	1850 ± 60	1080
0.8	2250 ± 50	890
1.6	3730 ± 70	540
2.0	4000 ± 120	500

obtained since the rate of triple helical formation (k_{on}) decreases with temperature leading to a positive slope ($-E_{on}/R$) of ln(k_{on}) versus $1/T$ (Supporting Information).[19] This is similar to the negative activation energies obtained for association of double and triple helical DNA complexes.[19,88] However, an elementary kinetic step cannot have an activation energy less than zero. Therefore, k_{on} (and k_{off}) must represent composites of rate constants for individual steps. As proposed for DNA·DNA complexes,[88] the negative activation energies rule out the formation of the first base pair as rate-limiting. The development of the nucleation-zipping model, as applied previously to triple helical DNAs,[19] can be used to explain this large negative value of E_{on}. The helix formation begins with two or three bases pairing and unpairing in rapid but unfavorable equilibrium. Upon formation of the critical intermediate, a helix nucleus is formed, which zips up to form the fully bonded helix more rapidly than it dissociates to single strands. The equilibrium constant $K = k_{on}/k_{off} = \beta s^n$, where β is the equilibrium constant for nucleation of the triplex (formation of the first base triplet). The chain growth parameter $s = k_f/k_b$, where k_f and k_b are the first-order rate constants for the formation and breakage of the base triplet at the end of a triplex segment, and n is the number of base triplets being formed. If ν is the number of base triplets in the nucleus, which is in rapid equilibrium with the separated duplex + third strand, the activation energy E_{on} equals the sum of one activation energy, E_{kf}, and $\nu + 1$ reaction enthalpies for base triplet reactions, $\Delta H_\beta + \nu\Delta H_s$:

$$E_{on} = E_{kf} + \Delta H_\beta + \nu\Delta H_s \qquad (2)$$

The first term is small and positive, but the enthalpies are negative, such that E_{on} becomes negative with its magnitude increasing with ν, and E_{off} ($E_{off} = E_{kf} - (n - \nu)\Delta H_s$) is largely positive.

While the E_{off} values do not show any significant change, within experimental error, for most aminoglycosides and polyamines, E_{on} values get more negative for neomycin (Table 6) and some aminoglycosides, suggesting an increased value of $\nu\Delta H_s$ (Supporting Information). Whether it is the increased number of bases required for nucleation (ν) or an increased $\Delta H_{s/base}$ that is responsible for the high E_{on} values is debatable; these numbers do suggest that neomycin is playing an active part in the rate-determining step-nucleation (zipping up) of the triplex. E_{on} values simply reflect the slope of the plot of the association rate constants (k_{on}) versus T (Supporting Information), which in turn reflects the change in A_{260} versus T observed in the annealing curve (Supporting Information). The sharper annealing curves (decreased hysteresis-Supporting Information) should then derive from a higher association rate constant of triplex formation. In the case of neomycin, the E_{off} values show a considerable increase initially (E_{off} = 122.1 kcal/mol at r_{db} = 0.26) which then decreases to 68.7 (r_{db} = 0.66). This behavior may reflect the nonspecific electrostatic stabilization of duplex and single strands at higher drug concentrations.

(7) Stabilization of RNA Triple Helices. Application of TFOs has mostly been in the regulation of transcription by binding of the TFO to duplex DNA in a sequence-specific manner. Thus, TFOs can compete with the binding of transcription factors to DNA and affect transcription initiation or elongation. However, single-stranded DNA or RNA can be targeted by an oligonucleotide, which can form both Watson−Crick base pairing and Hoogsteen base pairing with the target sequence. A foldback TFO (FTFO) and a circular TFO (CTFO) have been designed to bind to a single-stranded target sequence.[4,90−93] An increase in the specificity and affinity in the binding was observed.[92,93] When a single-stranded RNA is targeted, a FTFO or a CTFO can be utilized as an antisense oligonucleotide. In other applications, RNA can be used to target other duplexes such as double helical RNA, RNA hairpins, or RNA−DNA hybrids which are involved in biological processes. Thus, there has been considerable interest in the stability and specificity of recognition in triplexes consisting of both RNA and DNA strands.[36,94] Triplex formation at enzyme recognition sites may provide a means for specific control of enzymatic activity. Since the primary mode of interaction of aminoglycoside antibiotics has been their interaction with single-stranded

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Table 5. Rate Constants of Triplex Association (k_{on}) and Dissociation (k_{off}) and Free Energy of Formation of Poly(dA)·2Poly(dT) Triplex (15 μM/base, 0.15 M KCl) in the Presence of Different Amines and Aminoglycosides at 37 °C (Margin of Error: $k_{on} = \pm 10\%$; $k_{off} = \pm 10\%$; $\Delta G = \pm 10\%$, $T_m = \pm 1$ °C)

(37 °C)	4 μM, $r_{db} = 0.26$				10 μM, $r_{db} = 0.66$			
poly(dA)·2poly(dT) + aminoglycoside	k_{on} (M^{-1} s^{-1})	k_{off}*10^4 (s^{-1})	K_{eq}	ΔG (kcal/mol)	k_{on} (M^{-1} s^{-1})	k_{off}* 10^4 (s^{-1})	K_{eq}	ΔG kcal/mol
spermine (4)	11.9	2.4	0.74	0.2	140	2.0	10.2	−1.4
paromomycin (5)	2.3	15	0.02	2.2	10.1	5.8	0.26	0.8
lividomycin (5)	2.4	15	0.02	2.2	4.0	9.8	0.06	1.7
spermidine (3)	1.37	40.2	5×10^{-3}	3.1	0.74	56.0	2×10^{-3}	3.6
pentaethylenehexamine (6)	1.4	7.5	0.02	2.1	2.13	14.7	0.02	2.2

Table 6. Energies of Activation (E_{on}) and Dissociation (E_{off}) of Poly(dA)·2Poly(dT) Triplex (15 μM/Base, 0.15 M KCl) in the Presence of Increasing Concentration of Neomycin (Margin of error: $E_{on} = \pm 10\%$; E_{off} $\pm 10\%$)

poly(dA)·2poly(dT) + neomycin, μM	E_{on} (kcal/mol)	E_{off} (kcal/mol)
0	−58.0	62.3
1	−59.4	65.7
2	−62.1	82.1
4	−85.5	122.1
10	−111.0	68.3

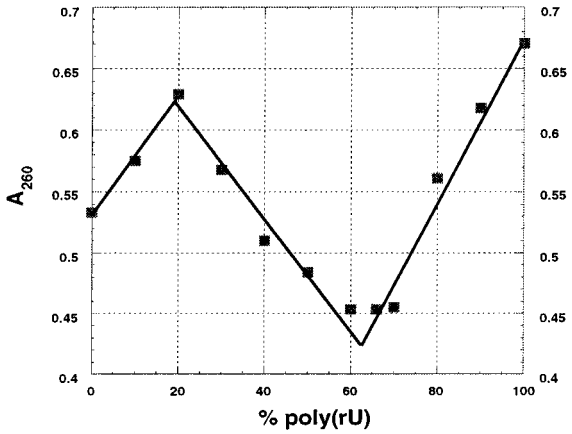

Figure 8. Job plot of poly(rA) (20 μM) and poly(rU) (20 μM) at 10 °C in the presence of 2.0 μM neomycin showing a minimum at 66% poly (rU).

RNA,[65−85,95−98] we turned our attention to investigate stabilization of RNA triple helices. Figure 8 shows the Job plot of poly(rA) and poly(rU) in the presence 2 μM ($r_{db} = 0.1$) neomycin. There is a minimum shift from 1:1 poly(rA):poly(rU) in the absence of drug (see Supporting Information) to 1:2 poly(rA)·2poly(rU) in the presence of 2 μM neomycin (Figure 8). In the presence of 2 μM neomycin, 100% poly(rA) shows some association {minimum at 100−90% poly (rA), Figure 8} which is diminished upon increasing the concentration of poly (rU). The triple helix is stabilized at $r_{db} = 0-0.5$, and at higher concentrations, the triplex and duplex transitions merge (Supporting Information). Among all of the aminoglycosides investigated (Table 7), neomycin, paromomycin, and gentamycin are the most active in stabilizing poly(rA)·2poly(rU) triplex ($r_{db} = 0-1$). (For the results of poly(rA)·2poly(rU) melting in the presence of these three aminoglycosides ($r_{db} = 0.025-1$), see

Table 7. Melting Temperatures of RNA {Poly(rA)·2Poly(rU)} Triplex and {Poly(rA)·Poly(rU)} Duplex at 260 nm at the Indicated Aminoglycoside Concentration[a]

poly(rA)·2poly(rU) + antibiotic	0.5 μM, $r_{db} = 0.025$		20.0 μM, $r_{db} = 1.00$	
	$\Delta T_{m3\rightarrow2}$	$\Delta T_{m2\rightarrow1}$	$\Delta T_{m3\rightarrow2}$	$\Delta T_{m2\rightarrow1}$
neomycin	4.9	1.0	49.0*	38.0*
paromomycin	2.6	0.3	29.0*	18.0*
lividomycin	2.9	0.0	26.0*	15.0*
kanamycin	1.9	0.1	14.9	11.7
gentamycin	4.0	0.6	33.2*	22.2*
sisomicin	1.7	0.4	33.0*	22.0*
tobramycin	1.9	0.9	32.0*	21.0*
amikacin	1.0	1.0	20.1*	9.1*
ribostamycin	1.9	0.1	15.9	15.0
streptomycin	0.9	0.0	6.9	6.0

[a] Asterisk(*) indicates $\Delta T_{m3\rightarrow1}$ {$\Delta T_{m3\rightarrow2}$ refers to $T_{m3\rightarrow1}$ ($r_{db} = 0-1$) − $T_{m3\rightarrow2}$ ($r_{db} = 0$)} and $\Delta T_{m2\rightarrow1}$ refers to $T_{m3\rightarrow1}$ ($r_{db} = 0-1$) − $T_{m2\rightarrow1}$ ($r_{db} = 0$)

Supporting Information). In the presence of 10 μM neomycin, the transition is from triplex to monomers ($T_{m3\rightarrow1}$), as evident from the melting curves at 260, 280 nm (Figure S9a,b, Supporting Information). The initial decrease in A_{280} refers to the formation of the triplex ($T_{m2\rightarrow3}$, similar to the absorption−temperature profile previously observed by Blake and Fresco at high salt concentrations),[99] which is then followed by the triplex melting to give single strands (the T_m values being the same at 260 and 280 nm). Since poly(rA)·2poly(rU) duplex transitions are not seen at 280 nm, absorbance changes at this wavelength are extremely useful for characterizing triplex transitions.[99−102]

Absorbance temperature profiles at 287, 284, and 280 nm were monitored for poly(rA)·2poly(rU) in the presence of 0.5 μM aminoglycoside (Figure S10, Supporting Information) to assign the triplex and duplex transitions (For ΔA signs at all wavelengths, see Supporting Information).[99]

Table 7 clearly shows that neomycin is the most active in stabilizing poly(rA)·2poly(rU) triplex as well ($\Delta T_{m3\rightarrow1} = 49.0$, $r_{db} = 1$). Spectinomycin was the only aminoglycoside that did not have any effect on the RNA triplex or duplex transitions under these conditions. Figure 9a,b depicts the ΔT_m values for poly(rA)·2poly(rU) triplex transitions in the presence of previously studied intercalators and minor groove binders (20 μM, $r_{db} = 1$). The presence of most intercalators leads to one transition at $r_{db} = 1$, whereas most groove binders show significant stabilization of triplex as well as duplex (Supporting Information). Clearly, neomycin is the most effective triplex stabilizer among all groove binders investigated. Its stabilization effect even surpasses all intercalators (except ellipticine) used in the study. These preliminary results indicate that neomycin

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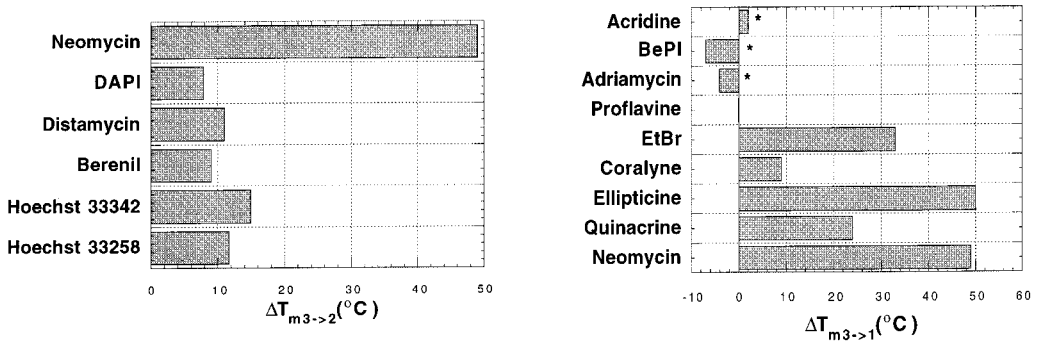

Figure 9. (a) Effect of 20.0 μM ($r_{db} = 1.0$) groove binders on the triplex melt of poly(rA)·2poly(rU). Neomycin and distamycin show $T_{m3\rightarrow1}$. (b) Effect of 20.0 μM ($r_{db} = 1.0$) intercalators on the triplex melt of poly(rA)·2poly(rU). Intercalators showing $T_{m3\rightarrow2}$ transition are designated by an asterisk (*).

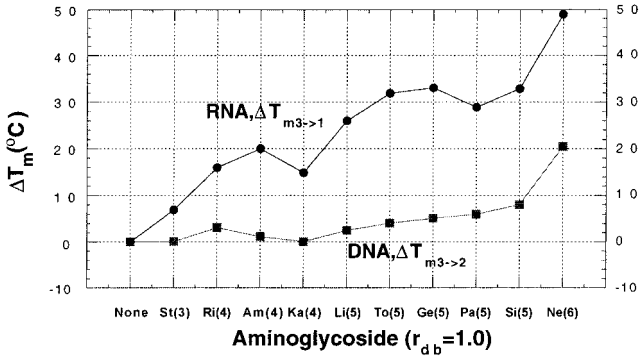

Figure 10. Plots of variation of DNA($\Delta T_{m3\rightarrow2}$) and RNA ($\Delta T_{m3\rightarrow1}$) triplex melting as a function of increasing charge (in aminoglycosides, $r_{db} = 1$). Aminoglycosides are written with the first two letters; the number of amines is shown in parentheses. RNA $\Delta T_{m3\rightarrow2}$ values are plotted for kanamycin, ribostamycin, and streptomycin.

can stabilize poly(rA)·2poly(rU) triplex at concentrations much lower than that needed for DNA triple helices (Table 7, Figure 10). Since RNA and DNA triple helices show two transitions ($T_{m3\rightarrow2}$ and $T_{m2\rightarrow1}$) at different salt concentrations, a direct comparison is not possible. A plot of ΔT_m ($\Delta T_{m3\rightarrow1}$ for RNA and $\Delta T_{m3\rightarrow2}$ for DNA) versus aminoglycosides (arranged in the order of increasing positive charge) is shown in Figure 10. The triplex melting points increase as the number of amines in the aminoglycosides increase (from left to right). RNA triplex ΔT_m values are, on average, 10−20 °C higher than ΔT_m for DNA triplex.

(8) Relative Toxicity of Aminoglycosides and Their Triplex Stabilization Effect: Is There a Correlation? It is believed that these aminoglycosides cause the formation of free radicals which lead to cell death.[103,104] Although all aminoglycosides have the potential for these toxic behaviors, they differ in their degree of toxicity in each of these target tissues. Neomycin is the most toxic of the aminoglycosides—it is primarily used for topical infections.[103,105] It is highly nephrotoxic and ototoxic and is by far the most potent in the area of neuromuscular blockade. Paromomycin differs from neomycin only in that it has one less amino group. However, this difference of one charge makes a great difference in the toxicity of the two compounds, as neomycin's overall toxicity, measured in median lethal dose (LD_{50}), or dose sufficient to kill half the test population, is much greater (LD_{50} of neomycin = 24, paromomycin = 160).[103,105] Although paromomycin is less toxic than neomycin, it is still so harmful that it, too, is rarely used. Lividomycin, which differs from paromomycin by an additional mannose, is much less toxic, with a LD_{50} of 280. Table 8 shows the order of acute LD_{50} values in mice, kidney, and neuromuscular toxicity and $\Delta T_{m3\rightarrow2}$ values for all aminoglycosides studied.[103,105,106] While neomycin is at the "head of the pack" with lowest LD_{50} value, the correlation of $\Delta T_{m3\rightarrow2}$ values versus LD_{50} values does not show a clear trend from Table 8. A better idea of the correlation becomes obvious when the aminoglycosides are studied on the basis of their structural family. This, we believe, is justified since the toxic effects and accumulation levels of these aminoglycosides in different tissue cells show a wide variation on the basis of their structure.[103,104] Neomycin, paromomycin, lividomycin, and ribostamycin (neomycin family) have a ribose that is attached to the neamine core. Table 9 lists these compounds with their LD_{50} and $\Delta T_{m3\rightarrow2}$ values. An increase in $\Delta T_{m3\rightarrow2}$ closely matches the decrease in LD_{50} values. Similarly Table 10 shows the other aminoglycosides (kanamycin and gentamycin families: kanamycin, gentamycin, amikacin,

Table 8. Toxicity Effects of Some Aminoglycosides in Kidney and Neuromuscular Blockade, the Acute LD_{50} Values in Mice, and Their Respective Effect on $\Delta T_{m3\rightarrow2}$ Values of DNA Triplex: Poly(dA)·2Poly(dT) at $r_{db} = 1.33$

antibiotic	kidney toxicity	neuromuscular blockade	LD_{50}	$\Delta T_{m3\rightarrow2}\, r_{db} = 1.33$
neomycin(6)	+++[a]	+++	24	24.7
paromomycin(5)	- -[b]	- -	160	8.5
lividomycin(5)	++	+++	280	3.0
kanamycin(5,4)	++	+++	206*[c]	3.1
gentamycin(5)	++	++	79	6.0
sisomicin(5)	++	++	34	9.1
tobramycin(5)	++	++	80	6.1
amikacin(4)	++	++	300	2.2
ribostamycin(4)	- -	- -	260	1.8
streptomycin(3)	+	++	300	−0.9

[a] +: indicates relative clinical importance of reaction. [b] - - : data not available. [c] *: the average value for kanamycin A(280) & B(132).

Table 9. Acute LD_{50} Values of Ribose-Linked Aminoglycosides (Neomycin Family) in Mice, and Their Respective Effect on $\Delta T_{m3\rightarrow2}$ Values of DNA Triplex: poly(dA)·2poly(dT) at $r_{db} = 1.33$

aminoglycoside	LD_{50}	$\Delta T_{m3\rightarrow2}\, r_{db} = 1.33$
neomycin(6)	24	24.7
paromomycin(5)	160	8.5
lividomycin(5)	280	3.0
ribostamycin(4)	260	1.8

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Table 10. Acute LD_{50} Values of Other Aminoglycosides (Kanamycin and Gentamycin Families) in Mice, and Their Respective Effect on $\Delta T_{m3\rightarrow 2}$ Values of DNA Triplex: Poly(dA)·2Poly(dT) at $r_{db} = 1.33$

aminoglycoside	LD_{50}	$\Delta T_{m3\rightarrow 2}\, r_{db} = 1.33$
sisomicin(5)	34	9.1
gentamycin(5)	79	6.2
tobramycin(5)	80	6.0
kanamycin(5,4)	205	3.1
amikacin(4)	300	2.2

sisomicin) that do not possess the ribose sugar. A good correlation exists between their LD_{50} and $\Delta T_{m3\rightarrow 2}$ values as well.

Our results suggest that an alternative mechanism of action of these antibiotics is indeed possible. The lethal doses and nephrotoxic effects of these antibiotics are in good match to the triplex stabilization properties observed. The positive charge of these aminoglycosides should allow them to cross cellular membranes where a significant accumulation is possible. Thus, inhibition of protein synthesis via H-DNA formation is a viable explanation for their toxic effects, in conjunction with previously proposed free radical-based mechanisms.[104]

Conclusions

The conclusions that can be drawn from our work are: (1) Neomycin is one of the most effective DNA triplex stabilization agents discovered to date; this is evident among polycationic/minor groove binders. and it also compares well in stabilization/selectivity to most intercalative agents. Neomycin can stabilize poly(dA)·2poly(dT) as well as a shorter, mixed pyrimidine base triplex without affecting the duplex. (2) Triplex stabilization is extremely sensitive to charge and charge placement. Development of novel synthetic aminoglycosides should help explore this sensitivity and further increase the effectiveness of neomycin and other aminoglycosides in stabilizing triple helical structures. (3) Triplex association rate constants can be significantly enhanced (10^3) by using aminoglycoside antibiotics—a crucial factor in potential therapeutic applications of TFOs. (4) The stabilization by neomycin is mainly due to increased k_{on} values, and the rate constants of dissociation (k_{off}) do not decrease to a large extent, leading to faster on-and-off rates for rapid equilibration to complementary target sequences. (5) Neomycin and other aminoglycosides can effectively stabilize RNA triplexes at concentrations much lower than needed for DNA triplex, neomycin being the best RNA triplex stabilizer among all groove binders and most intercalators, and (6) There exists a clear correlation between the toxicity of these antibiotics and their ability to stabilize DNA triple helix, suggesting that these antibiotics may be able to aid H-DNA formation in vivo and could have an alternative mode of action that has been previously unexplored.

Note Added after ASAP: An invalid version of Table 4 was posted ASAP May 16, 2001; the corrected version was posted May 18, 2001.

Supporting Information Available: Intercalator and minor groove binding structures; Arrhenius plots, annealing, and melting curves; Tables for E_a, k_{on}, k_{off} for triplex formation in the presence of different aminoglycosides, amines at 4 and 10 μM; T_m values for ligands used in DNA and RNA studies, UV decay curves, and ΔA signs for poly(rA)·2poly(rU) triplex in the presence of different ligands (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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