

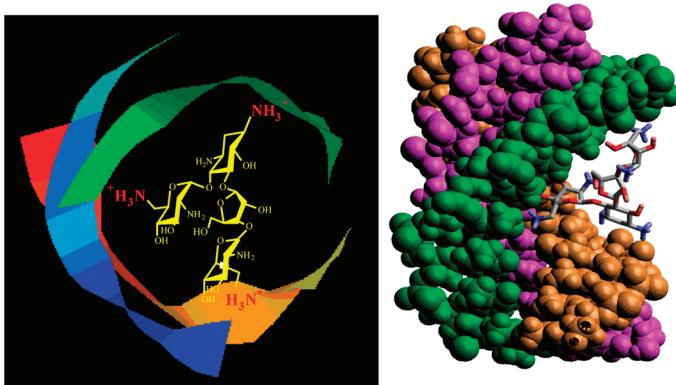
New Approaches Toward Recognition of Nucleic Acid Triple Helices

DEV P. ARYA*

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

RECEIVED ON AUGUST 12, 2010

CONSPECTUS



ADNA duplex can be recognized sequence-specifically in the major groove by an oligodeoxynucleotide (ODN). The resulting structure is a DNA triple helix, or triplex. The scientific community has invested significant research capital in the study of DNA triplexes because of their robust potential for providing new applications, including molecular biology tools and therapeutic agents. The triplex structures have inherent instabilities, however, and the recognition of DNA triplexes by small molecules has been attempted as a means of strengthening the three-stranded complex. Over the decades, the majority of work in the field has focused on heterocycles that intercalate between the triplex bases. In this Account, we present an alternate approach to recognition and stabilization of DNA triplexes.

We show that groove recognition of nucleic acid triple helices can be achieved with aminosugars. Among these aminosugars, neomycin is the most effective aminoglycoside (groove binder) for stabilizing a DNA triple helix. It stabilizes both the TAT triplex and mixed-base DNA triplexes better than known DNA minor groove binders (which usually destabilize the triplex) and polyamines. Neomycin selectively stabilizes the triplex (TAT and mixed base) without any effect on the DNA duplex. The selectivity of neomycin likely originates from its potential and shape complementarity to the triplex Watson–Hoogsteen groove, making it the first molecule that selectively recognizes a triplex groove over a duplex groove. The groove recognition of aminoglycosides is not limited to DNA triplexes, but also extends to RNA and hybrid triple helical structures.

Intercalator–neomycin conjugates are shown to simultaneously probe the base stacking and groove surface in the DNA triplex. Calorimetric and spectroscopic studies allow the quantification of the effect of surface area of the intercalating moiety on binding to the triplex. These studies outline a novel approach to the recognition of DNA triplexes that incorporates the use of noncompeting binding sites. These principles of dual recognition should be applicable to the design of ligands that can bind any given nucleic acid target with nanomolar affinities and with high selectivity.

1. Introduction

Out of the earth shall come thy salvation.

Selman Waksman

Duplex DNA can be sequence-specifically recognized by small molecules such as minor groove binders and larger

molecules such as oligodeoxynucleotides (ODNs, major groove binders).^{1,2} Major groove recognition of the duplex DNA by ODNs results in a triple helix in a parallel or antiparallel form (Figure 1). Triple helix formation was first reported in 1957 by Felsenfeld and co-workers.^{3,4} The elucidation of the DNA duplex structure at the same time

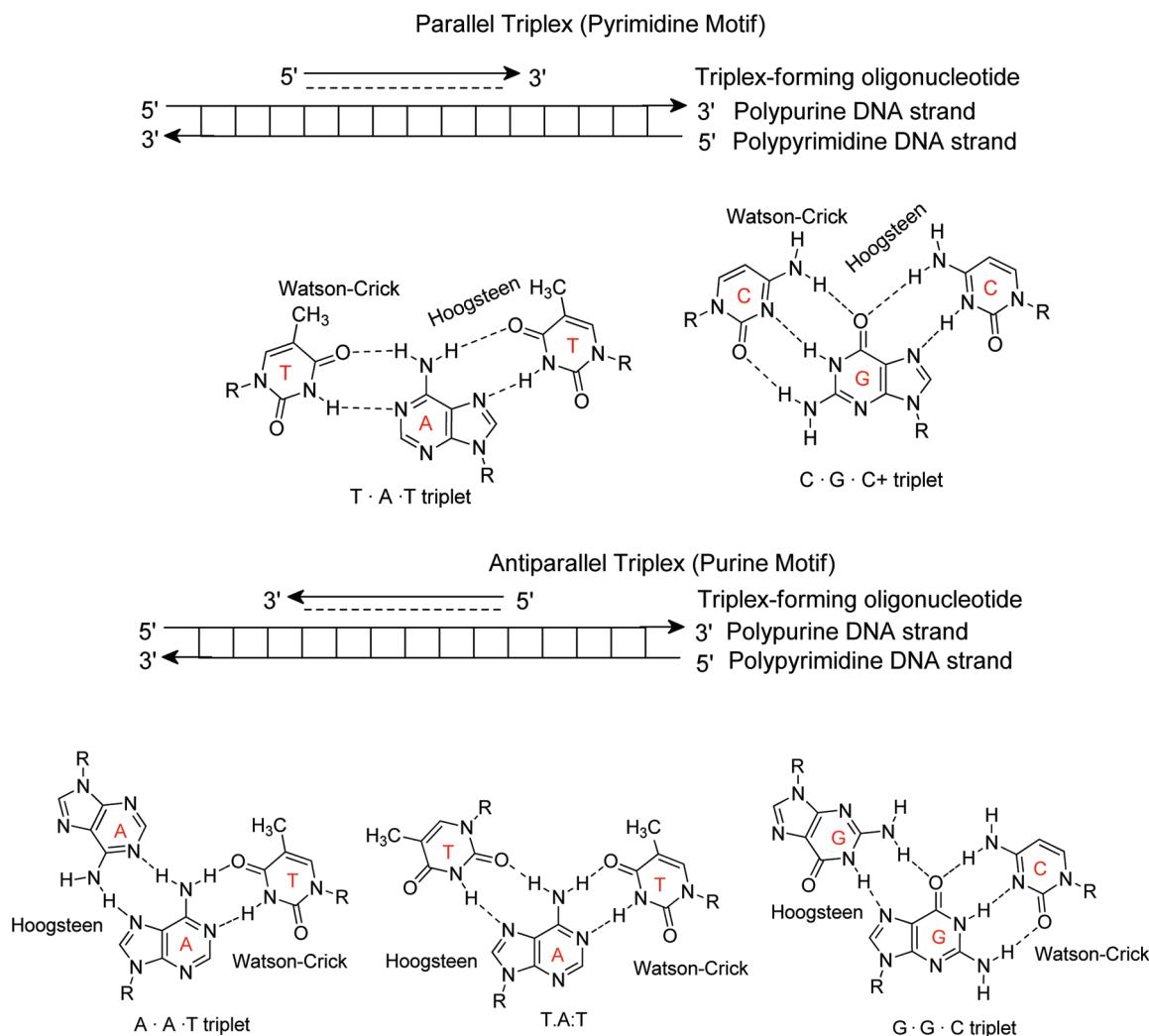


FIGURE 1. Base interactions in parallel (pyrimidine motif, top) and antiparallel (purine motif, bottom) triple helices. These are defined with respect to the orientation of the TFO and homopurine Watson–Crick (W–C) strand.

kept the limelight away from the three-stranded DNA structures until the 1980s. Eventually, work by Dervan, Hélène, and others showed the broad potential of triplex-forming ODNs in targeting duplex and single-stranded DNA and in inhibiting sequence-specific DNA–protein interactions.^{5–9} DNA triplex formation has continued to garner much interest in the scientific community because of the possible applications in developing new molecular biology tools as well as therapeutic agents.^{10–13} Specific inhibition of transcription has been induced via triplex formation at poly-(purine/pyrimidine) sites in promoter sequences. These and numerous other findings that support the feasibility of an antigene approach for therapeutically regulating specific gene expression have been discussed in a number of reports.^{14–16} A recent report has postulated that DNA triplexes may even be present at the active site of the DNA polymerases.¹⁷

Several intercalators as well as various DNA minor groove ligands have previously been shown to bind to DNA triple helices.^{18,19,19–21} Intercalators usually stabilize to a greater extent triple helices containing TAT triplets, whereas minor groove binders usually destabilize triplexes.²² At the beginning of this millennium, when we began our work on recognition of nucleic acids, one surprising fact stared at us: While there were a number of intercalators that stabilized DNA duplex and triplex structures (selectively and nonselectively), there were no examples of groove-binding ligands that selectively recognized the triple helices (spermine,²³ with its flexible amines as the possible weak exception). This was in stark contrast to the numerous sequence-specific groove binders known to bind in the minor groove of duplex DNA.^{2,24,25} Time and again, one has had to look to natural products to deal with such problems in recognition of biomolecules. Examples of such

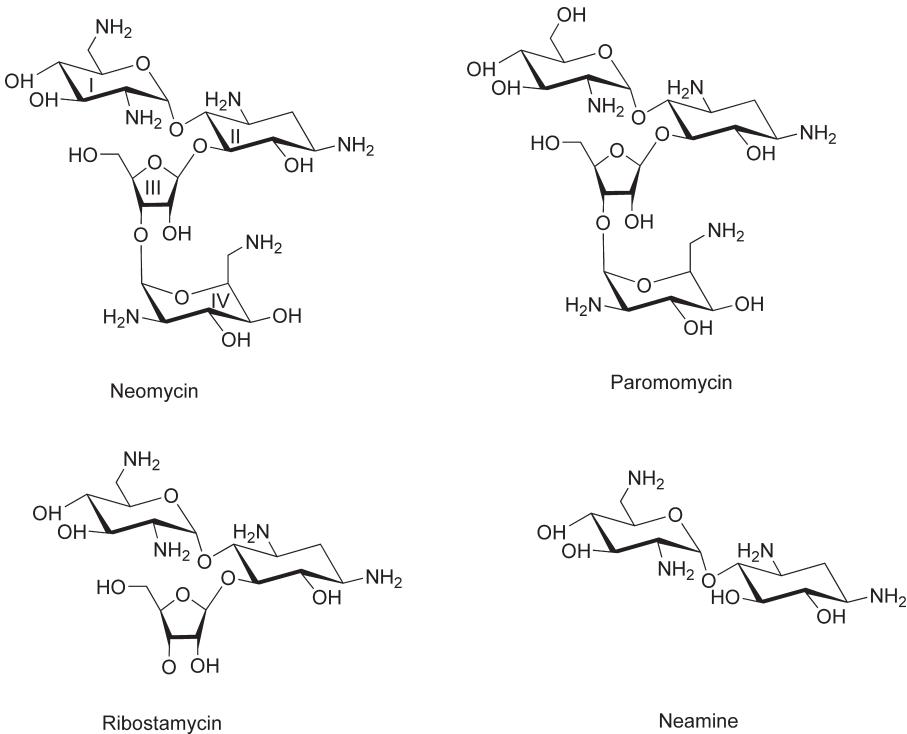


FIGURE 2. Structures of some aminoglycoside antibiotics. Ring numbering scheme is shown for neomycin.

natural products include crescent shaped netropsin and distamycin^{26–28} which were key leads in the design principles that are used to target the DNA minor groove today. In such a quest for ligands for triple-helix-specific stabilization, we decided to investigate aminoglycoside antibiotics, the antibacterial scaffolds first discovered by Waksman in 1944 for activity against tuberculosis (Figure 2).^{29–32} In this Account, I will present neomycin as one of the first examples that bridge the gap in molecular recognition of triplex structures and increase our understanding of the recognition principle(s) involved in selective targeting of nucleic acid triplex grooves.

2. Thermal Denaturation Studies and Aminoglycoside Structure–Activity Relationships

Thermal denaturation studies of triplexes formed from ODNs as well as polynucleotides were carried out in the presence of aminoglycosides, using UV spectroscopy at 260/280 nm. These studies showed the remarkable effectiveness of neomycin in stabilizing the triplex without affecting the duplex T_m .³³

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 { $T_{m3 \rightarrow 2}$ (triplex melting point)} = 34 °C and $T_{m2 \rightarrow 1}$

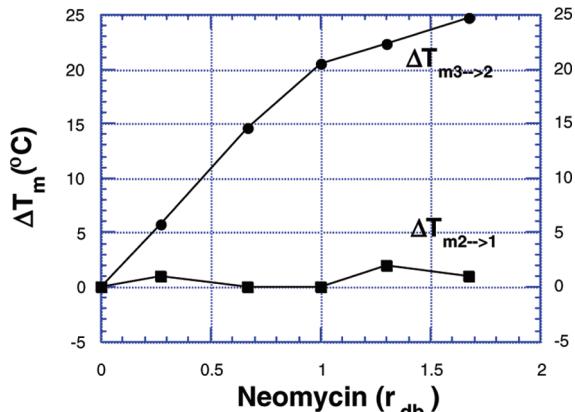


FIGURE 3. Plots of variation of triplex melting ($T_{m3 \rightarrow 2}$) and duplex melting ($T_{m2 \rightarrow 1}$) of poly(dA)·2poly(dT) as a function of increasing neomycin concentration { r_{db} = drug(neomycin)/base triplet ratio}. Conditions: 10 mM sodium cacodylate, 150 mM KCl, pH 6.8, [poly(dA)·2poly(dT)] = 15 μ M/base triplet. Reprinted with permission from ref 33. Copyright 2010 Elsevier.

(duplex melting point) = 71 °C, r_{db} = 0.15 (ratio of drug(neomycin)/base triplet). Triplex stabilization was found to be dependent on neomycin concentration. Figure 3 shows that, by increasing the molar ratios of neomycin from 0 to 25 μ M, r_{db} = 0–1.67, the triplex melting point is increased by close to 25 °C, whereas the duplex is virtually unaffected.

Nature has provided us antibiotics to allow for a simplified version of aminoglycoside–triplex structure–activity

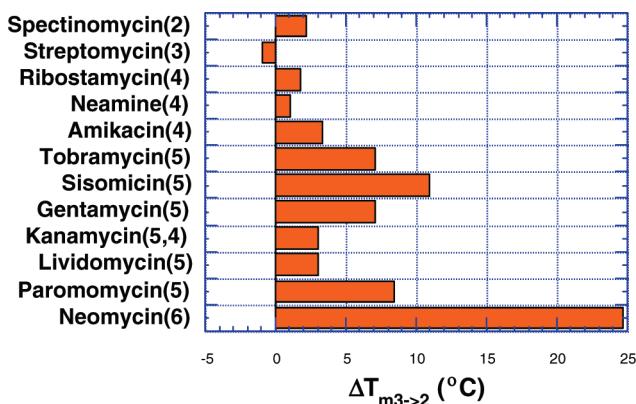


FIGURE 4. Effect of aminoglycoside antibiotics on the melting of poly(dA)·2poly(dT) triplex ($r_{db} = 1.67$). Solution conditions: 150 mM KCl, 10 mM sodium cacodylate, pH 6.8, [poly(dA)·2poly(dT)] = 15 μ M/base triplet. Number of amines in each antibiotic is shown in parentheses. Reprinted with permission from ref 34. Copyright 2001 American Chemical Society.

TABLE 1.³⁵ Fluorescence Derived AC₅₀ Values for Aminoglycoside Binding to 5'-dA₁₂-x-dT₁₂-x-dT₁₂-3' Triple Helix at pH 5.5 and 6.8 in 10 mM Sodium Cacodylate, 0.5 mM EDTA, and 150 mM KCl^a

| aminoglycoside | AC ₅₀ (μ M) | |
|----------------|-----------------------------|--------|
| | pH 6.8 | pH 5.5 |
| neomycin | 35.5 | 13.3 |
| paromomycin | 179.0 | 157.9 |
| ribostamycin | 486.0 | 459.0 |

^aT = 10 °C, [DNA triplex] = 100 nM/strand, [thiazole orange] = 700 nM.

relationship (Figure 4). A few of these aminosugars can be viewed as simple variations of neomycin pharmacophore with functional group deletions. Most aminoglycosides with five or more amines are able to stabilize the triple helix (increasing $\Delta T_{m3 \rightarrow 2}$, without significantly affecting the $\Delta T_{m2 \rightarrow 1}$ values, Figure 4). The structural difference between paromomycin and neomycin is a positively charged amino group (present in neomycin), replacing a neutral hydroxyl (present in paromomycin). This leads to a difference of 16 °C in $T_{m3 \rightarrow 2}$ values ($r_{db} = 1.67$) at 150 mM K⁺ concentration. As also seen from Figure 4, neomycin is far more effective than paromomycin or ribostamycin in stabilizing triple helices.³⁴ Since both paromomycin and ribostamycin can be derived by deletion of specific groups/rings in neomycin, these two aminoglycosides offer preliminary evidence for specific functional group (ring I amine) or ring (ring IV) participation in DNA triplex binding by neomycin.

A fluorescent intercalator displacement (FID) assay was then used to determine the AC₅₀ values for the three aminoglycosides with poly(dA)·2poly(dT) and an intramolecular 5'-dA₁₂-x-dT₁₂-x-dT₁₂-3' (x = hexaethyleneglycol

linker) triplex. These studies have been recently published, and the results are summarized below.³⁵ The AC₅₀ values reported in Table 1 are the aminoglycoside concentrations required to displace 50% of thiazole orange from the triplex, as measured by a decrease in 50% of the fluorescence. At pH 6.8, the neomycin AC₅₀ (AC₅₀ = 35.5 μ M) is 6-fold lower than that of paromomycin (AC₅₀ = 179 μ M) and approximately 14-fold lower than that of ribostamycin (AC₅₀ = 486 μ M). A similar trend, with neomycin (AC₅₀ = 13 μ M) < paromomycin (AC₅₀ = 157 μ M) < ribostamycin (AC₅₀ = 459 μ M) is observed at pH 5.5.

Studies with the poly(dA)·2poly(dT) triplex showed the same trends in AC₅₀ values. When the FID experiments were carried out with poly(dA)·poly(dT) duplex, thiazole orange could not be displaced by up to 10 mM neomycin, suggesting that the affinity of the aminoglycoside is 2–3 orders of magnitude lower for the AT rich DNA duplexes, when compared to its affinity for the AT rich DNA triplex.

These results suggested that (i) paromomycin is much weaker in stabilizing the triplex (amino group in ring I is a key element in DNA triplex recognition); (ii) ribostamycin and neamine are much weaker in stabilizing the triplex, and both have similar effect on triplex stabilization (ring IV amines are involved in recognition; and ring III likely provides the neomycin conformer necessary for successful binding).

3. Stabilization of DNA Triple Helix Poly(dA)·2poly(dT) by Other Ligands³⁴

A comparison with DNA groove binders indicates that neomycin is much more active than the minor groove binders (Figure 5) in stabilizing the triplex. The minor groove binders, in general, are not triplex-specific (berenil, distamycin, Hoechst dyes), and some are known to even destabilize the triplex (berenil, distamycin) because of their preference for the DNA duplex.³⁴ The minor groove binders such as Hoechst 33258 stabilize the DNA duplex much better than the DNA triplex, suggesting that the significant perturbations take place in the minor groove upon ODN binding to the duplex major groove. Binding studies suggest that neomycin, as opposed to minor groove DNA duplex binders, can preferentially bind the DNA triplex grooves over those present in the AT rich DNA duplex. Structural data identifying specific groove–drug interactions would further clarify which of the three triplex grooves are being targeted. The intercalating ligands, on the other hand, are equally or more effective in stabilizing the triple helix at low concentrations.³⁴ At higher concentrations, the intercalating ligands

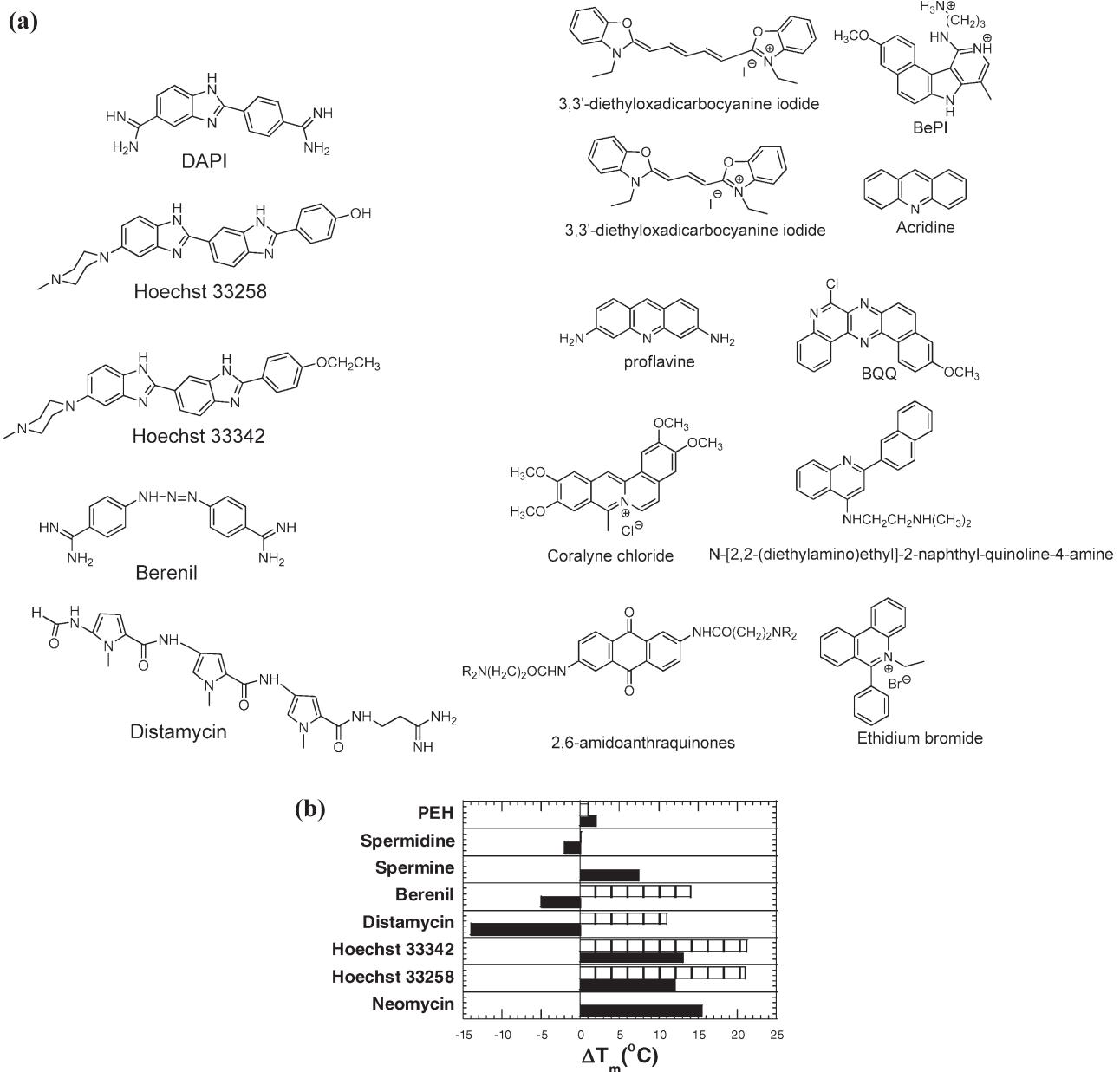


FIGURE 5. (a) Structures of groove binders (left) and intercalators (right), known to bind to DNA tripleplexes. (b) Effect of $10\ \mu\text{M}$ ($r_{db} = 0.66$) groove binders on the DNA tripleplex melt ($\text{poly(dA)} \cdot 2\text{poly(dT)}$) (black) and the duplex melt ($\text{poly(dA)} \cdot \text{poly(dT)}$) (boxed). Distamycin does not show a $T_{m3 \rightarrow 2}$ transition ($<20^\circ\text{C}$). PEH = pentaethylene hexamine. Solution conditions: $150\ \text{mM}$ KCl, $10\ \text{mM}$ sodium cacodylate, pH 6.8, DNA = $15\ \mu\text{M}$ /base triplet. Reprinted with permission from ref 34. Copyright 2001 American Chemical Society.

begin to stabilize the duplex as well, though significant advances have recently been reported in the design of triplex selective intercalators.^{19,20,36,37}

The planar moieties do offer an advantage over the aminoglycosides in terms of cellular permeability and nuclear localization. Based on the limited data that is available, aminoglycosides do not enter mammalian cells readily, whereas dyes such as Hoechst 33342 are known to rapidly enter the nucleus. Models for bacterial cell permeability by aminoglycosides have been proposed and discussed in a

recent review.²⁹ However, minor modifications in aminoglycosides can dramatically alter their permeability³⁸ and should therefore not detract from the development of aminoglycosides in targeting mammalian cells.

As previously discussed, the minor groove (W–C groove) of the triplex is similar in width and depth to that of B-type duplex DNA, yet the two grooves are not identical.^{39–41} The perturbation upon major groove binding of the third strand leads to a difference in overall geometry. The B-form duplex minor groove has a U-shaped cross section, whereas

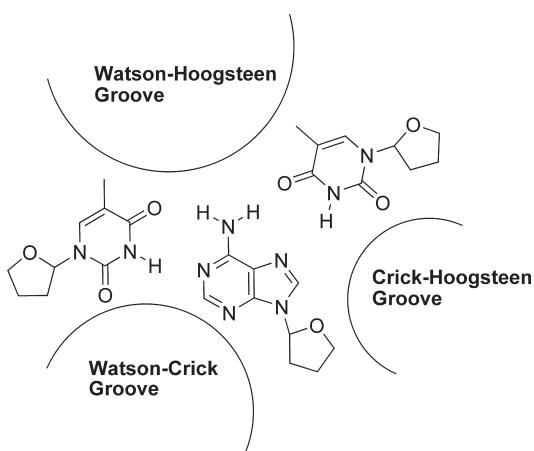


FIGURE 6. Structure of a TAT DNA triplex showing different grooves. W–H groove is formed between two pyrimidine strands.^{42–44}

the triplex minor groove adopts a V-shaped cross section. Duplex minor groove binding ligands such as netropsin and distamycin can bind to triple helices, but they destabilize them as they preferentially bind to the duplex minor groove generated on loss of the triplex-forming strand.^{22,34}

The triplex major groove, on the other hand, is quite different from that in standard B-type DNA, since the presence of the third strand widens the major groove and divides it into two asymmetric parts: the smaller Crick–Hoogsteen (C–H) groove and the wider Watson–Hoogsteen (W–H) groove (Figure 6). The W–H groove is wide enough to interact with such molecules as neomycin and, in contrast to the smaller grooves, is more flexible. This flexibility is likely used by the DNA triplex to adapt its shape to an aminosugar such as neomycin. These results are consistent with W–H groove being the likely site for selective triplex stabilizers, as opposed to the DNA duplex selective minor groove binders, although structural evidence is still needed to verify this hypothesis.

4. A Proposed Model for Neomycin Binding to the DNA Triplex Watson–Hoogsteen Groove^{21,22}

To build a model for neomycin binding to the major groove of the DNA triplex, circular dichroism (CD) spectroscopic studies, pH dependence, salt dependence, and viscosity measurements were performed. A full thermodynamic profile of aminoglycoside complexation with DNA tripleplexes detailing these studies has recently been published.³⁵

Key results from these studies are mentioned below. (a) The binding constant (10^5 – 10^6 M^{-1}) decreases with increasing salt concentration, and a plot of $\log K$ versus $\log (K^+)$ shows that

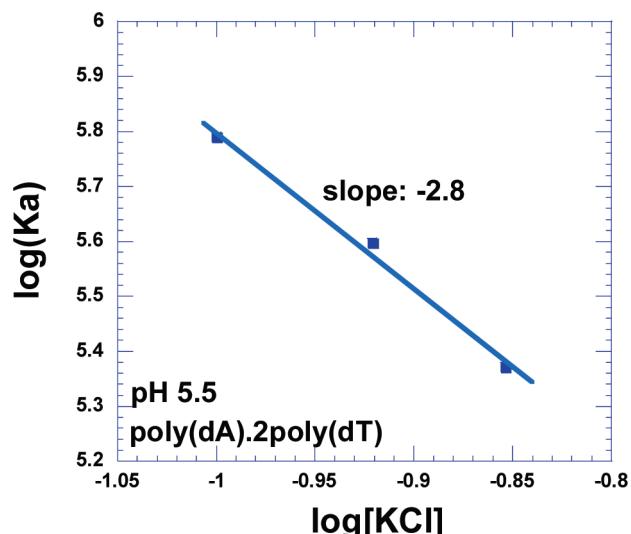


FIGURE 7. Salt dependence of the neomycin binding with poly-(dA)-2poly(dT) triplex in 10 mM sodium cacodylate, 0.5 mM EDTA, and pH 5.5. $T = 10^\circ\text{C}$. The experimental data were fit with linear regression, and the solid line reflects the resulting curve fit. Reprinted with permission from ref 35. Copyright 2010 Elsevier.

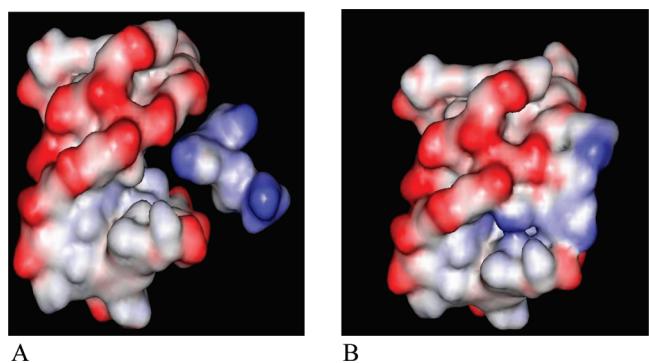


FIGURE 8. Electrostatic surface potential maps of (A) neomycin approaching the W–H groove of the triplex and (B) neomycin buried in the triplex groove. Reprinted with permission from ref 45. Copyright 2001 American Chemical Society.

approximately three ion pairs are formed between neomycin and the DNA triplex (Figure 7). (b) The viscosity of poly(dA)-2poly(dT) triplex with increasing concentrations of neomycin was also investigated to understand the mode of drug interaction. Intercalating compounds, upon binding to the nucleic acid, lead to an increased solution viscosity as the length of the rod (DNA polymer) increases. Neomycin, like other groove binding drugs, on the other hand, leads to a decrease in viscosity upon its interaction with the poly(dA)-2poly(dT) triplex,⁴⁵ suggesting groove binding.

Modeling studies suggest that the shape and potential complementarity of neomycin to the W–H groove is largely responsible for its selectivity in triplex recognition (Figure 8).⁴⁵ The selective binding of neomycin to the triplex

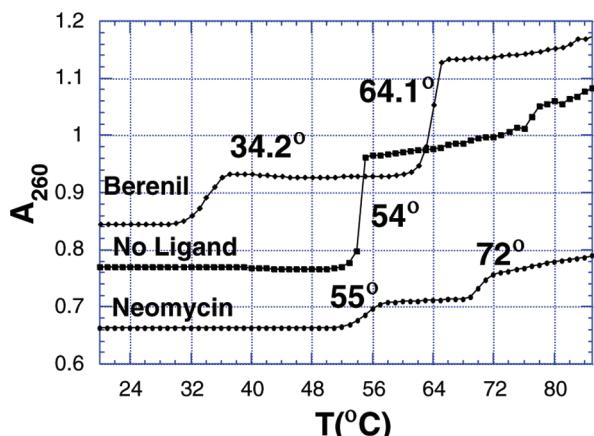


FIGURE 9. Melting curves for poly(rA)·2poly(dT) complex showing ligand ($20\ \mu\text{M}$) induced triplex formation in the presence of berenil and neomycin. Solution conditions: rA, $30\ \mu\text{M}/\text{base triplet}$; $18\ \text{mM}\ \text{NaCl}$; $10\ \text{mM}$ sodium cacodylate; $0.1\ \text{mM}\ \text{EDTA}$, pH 6.8. Reprinted with permission from ref 49. Copyright 2001 American Chemical Society.

is achieved in the presence of physiological salt (100–150 mM KCl). In the absence of salt, one observes indiscriminate binding to duplex/triplex backbones,³⁴ suggesting the importance of shape and potential complementarity of neomycin to DNA triplex as a key factor in recognition (in the presence of KCl).

5. Stabilization of Oligomeric, Mixed Base, and Non-DNA Triple Helices

Neomycin also stabilizes oligomeric TAT triplexes that do not form spontaneously, in addition to mixed base sequences. As seen in the thermal denaturation studies of a dA₁₆·2dT₁₆ triplex,⁴⁵ in the absence of drug, a transition for this triplex is not observed (150 mM KCl, pH = 6.8, 10 mM sodium cacodylate, 0.5 mM EDTA, UV or CD melts), even when the dT₁₆ and dA₁₆ strands are mixed in a 2:1 ratio. Upon addition of small amounts of neomycin, a clear T_{m3-2} transition becomes visible at 31 °C, while the duplex transition is not affected. Thermal denaturation studies of mixed base tripleplexes such as the 22-mer triplex (below) in the presence of neomycin were found to stabilize the triplex without any effect on the duplex at a pH of 6.8.



Stabilization of RNA and DNA.RNA Triple Helices. Single-stranded DNA or RNA can be targeted by an ODN, which

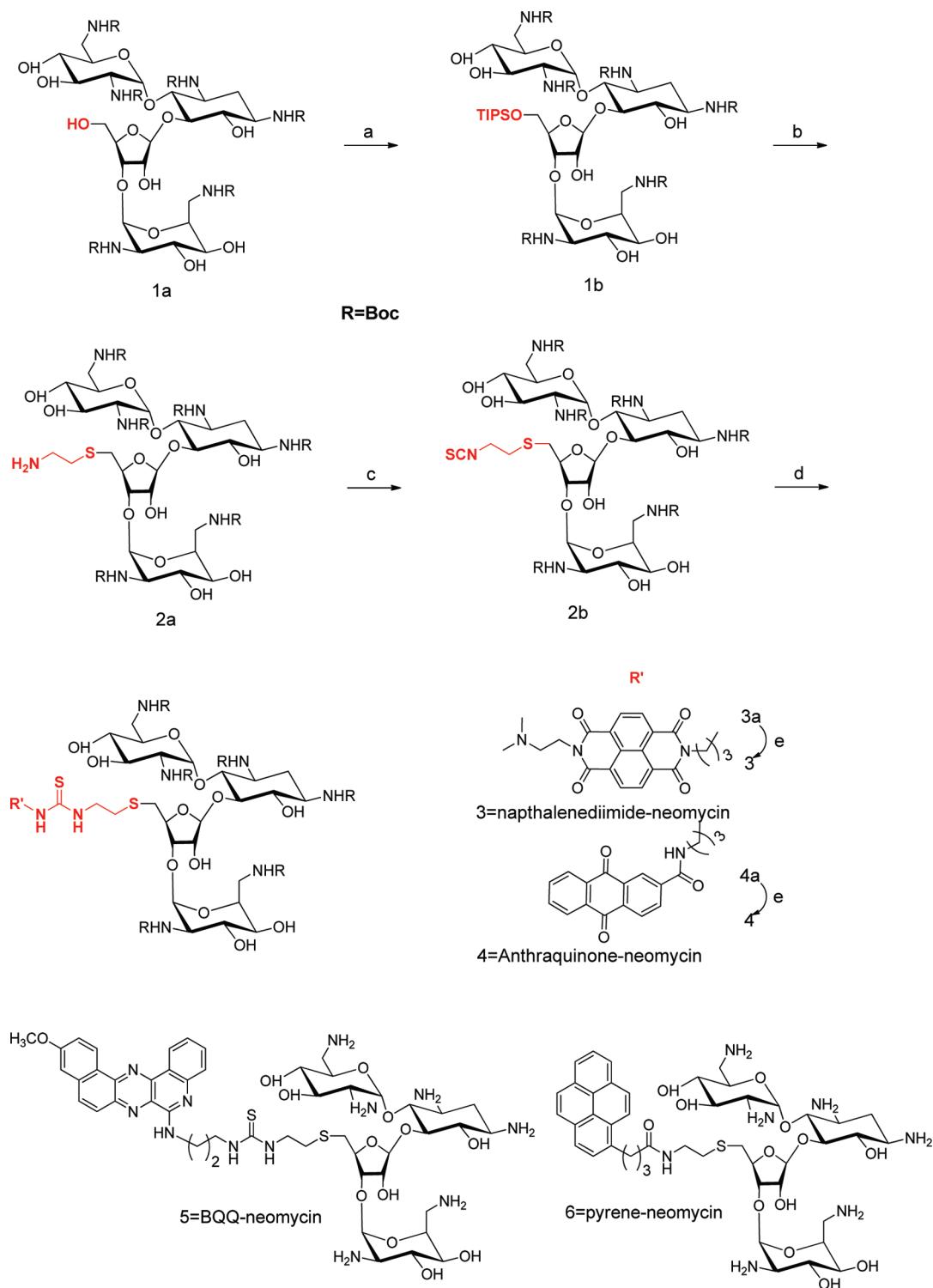
can form both Watson–Crick base pairing and Hoogsteen base pairing with the target sequence.^{1,10,46–48} Among all the aminoglycosides investigated, neomycin, paromomycin, and gentamycin were found to be the most active in stabilizing poly(rA)·2poly(rU) triplex ($r_{db} = 0-1$) and neomycin is the most active in stabilizing poly(rA)·2poly(rU) triplex as well.³⁴ Similar to the DNA triplex, the RNA triplex melting points increase as the number of amines in the aminoglycosides increase.³⁴ These trends of higher affinity of aminoglycosides to RNA tripleplexes are also observed in FID and calorimetric assays (unpublished results).

Neomycin has also been shown to induce the formation of a poly(rA)·2poly(dT) triplex ($T_{m3-2} = 53.2$) much more effectively than previously reported ligands (berenil, $T_{m3-2} = 34.2$). Without any ligand, only the duplex transition is seen (Figure 9). A similar effect has been reported with the 2poly(rA)·poly(dT) triplex.⁴⁹ As mentioned with the RNA triplex, FID and Isothermal Titration Calorimetry (ITC) results show that, for AT/U rich nucleic acids, affinity of neomycin follows the trend: DNA duplex < DNA triplex < DNA.RNA hybrids < RNA duplex ~ RNA triplex.

6. Molecular Recognition of DNA Triple Helices by Dual Recognition Conjugates

With synthetic modifications, it is now possible to design ligands that can even compete with aminoglycoside binding to their natural targets, duplex RNA. The triplex-specific interactions (at physiological salt concentrations) of neomycin can be combined with intercalating agents to lead to an overall stabilization of the triplex over the parent duplex.

a. Synthetic Approach toward the Development of Neomycin–Intercalator Conjugates⁵⁰ The synthesis of conjugates **3–6** adopts a uniform strategy, in which the 5'-OH position of neomycin (highlighted in red) was selected to tether the intercalators because conjugation at 5'-OH leaves available all aminoglycoside amines necessary for binding to the triplex. The successful approach to synthesize a neomycin derivative (**1a**) that was further used for conjugation with intercalator amines involved conversion of the 5'-OH group of **1a** into a good leaving group (triisopropylbenzenesulfonyl, TPS) in **1b**, followed by reaction with 1,2-aminoethanethiol to yield **2a**, which further reacted with 1,1'-thiocarbonyldi-2-(1H)-pyridone (TCDP) to convert the amino group into an electrophile (isothiocyanate) (Scheme 1). Subsequently reacting **1b** with intercalator amines yielded **3a–6a**. Treatment of **3a–6a** with trifluoroacetic acid (TFA) removed the acid labile Boc-protecting groups to produce **3–6** in quantitative yields. Intermediate **2b** has broad applications in terms of conjugation of neomycin

SCHEME 1.^a

^a(a) Triisopropylbenzenesulfonyl chloride, pyridine, r.t.; (b) 1,2-Aminoethanethiol·HCl, Na, and EtOH; (c) TCDP, DCM, DMAP, r.t.; (d) intercalator-amine, DMAP, DCM; (e) TFA/CH₂Cl₂. The site of aminoglycoside modification is shown in red.

with other moieties containing amino groups and has been used to conjugate aminoglycosides to numerous nucleophiles (Scheme 1).^{51–54}

b. Thermodynamics of Dual Recognition: DNA Triplex Interactions. The binding preference of intercalator-neomycin conjugates (**3–6**) to poly(dA)·2poly(dT) was

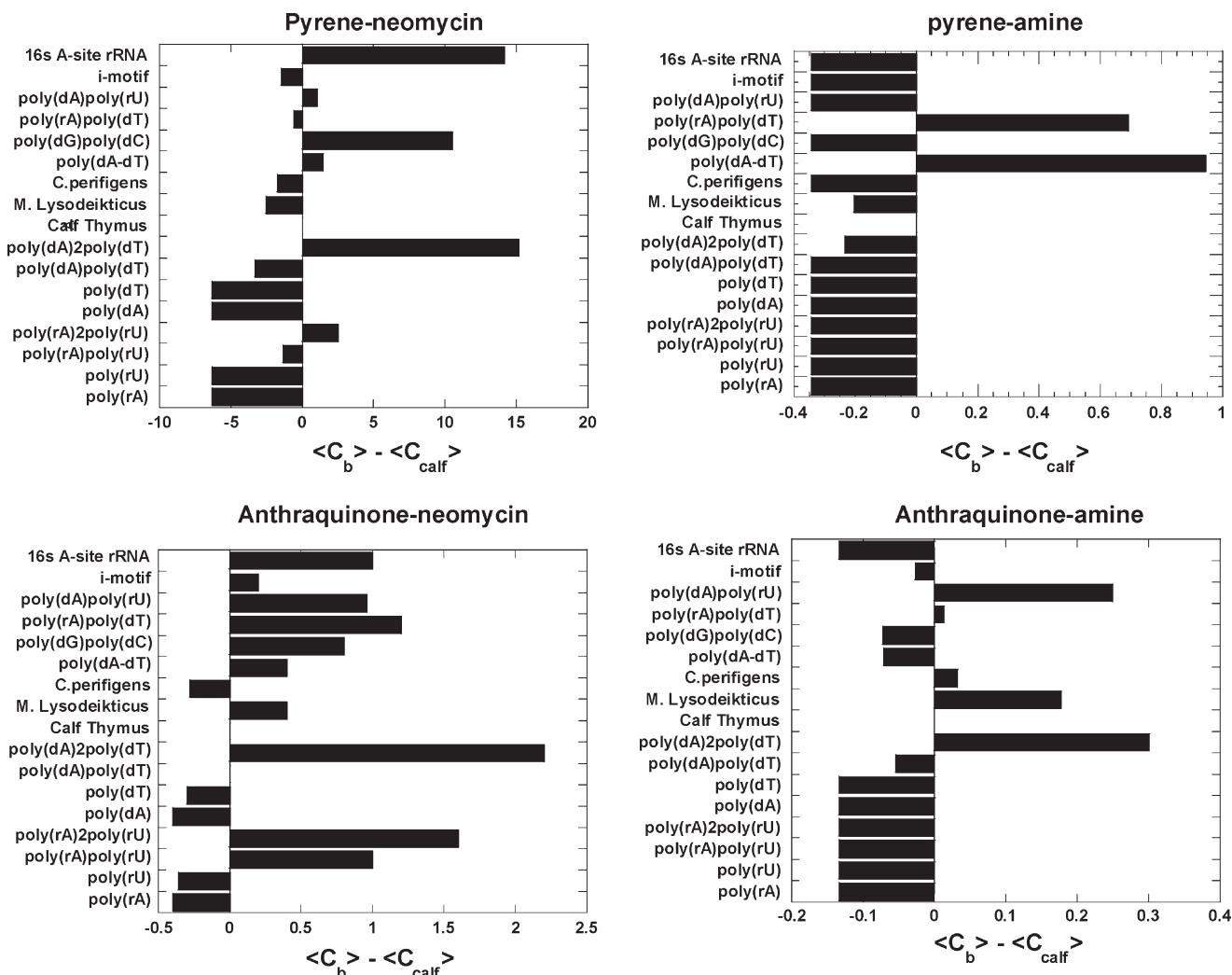


FIGURE 10. Competition dialysis results of pyrene-neomycin and anthraquinone-neomycin as well as their corresponding intercalators pyrene-amine and anthraquinone-amine ($1 \mu\text{M}$) with various types of nucleic acids ($75 \mu\text{M}$). Buffer: Na_2HPO_4 (6 mM), Na_2PO_4 (2 mM), Na_2EDTA (1 mM), NaCl (185 mM), and pH 7.0. X-axis shows concentration of bound drug (C_b) – concentration of drug bound to calf thymus DNA (C_{calf}). Reprinted with permission from ref 50. Copyright 2010 American Chemical Society.

TABLE 2. Thermodynamic Profiles of Neomycin and Intercalator-Neomycin Conjugates (**3–6**) with Poly(dA)·2poly(dT) at pH 5.5^a

| ligand | ΔC_p (cal/mol·K) | $K_{T(20^\circ\text{C})} (\text{M}^{-1})$ |
|------------------------------------|--------------------------|---|
| neomycin | -87 ± 4 | $(2.4 \pm 0.1) \times 10^5$ |
| 6 (pyrene-neomycin) | -10 ± 3 | $(1.9 \pm 0.1) \times 10^6$ |
| 3 (naphtheneimide-neomycin) | -40 ± 20 | $(5.5 \pm 0.3) \times 10^6$ |
| 4 (anthraquinone-neomycin) | -110 ± 15 | $(3.7 \pm 0.1) \times 10^7$ |
| 5 (BQQ-neomycin) | -220 ± 20 | $(2.7 \pm 0.3) \times 10^8$ |

^aExperimental conditions: sodium cacodylate (10 mM), EDTA (0.5 mM), KCl (150 mM).⁵⁰

confirmed by the competition dialysis experiments (Figure 10). Results (Figure 10) showed that all conjugates **3–6** showed selectivity toward the DNA triplex. Binding of conjugates **3–6** to the DNA triplex was comparable or even higher than that of the natural aminoglycoside target, the RNA A-site.

Pyrene-neomycin **6** and anthraquinone-neomycin **4** bind to the poly(dG)·poly(dC) duplex, while the isolated intercalators do not show any binding to this duplex. We have previously shown that neomycin binds to A-form nucleic acids,⁵⁵ such as the poly(dG)·poly(dC) duplex, and the competition dialysis studies here confirm our previous findings.

Thermodynamic studies were then conducted at pH 5.5 to minimize the heats of binding induced protonation of amino groups in neomycin, allowing the estimation of intrinsic heats of binding.

The binding affinity of all tested ligands with poly(dA)·2poly(dT) increased in the order neomycin < **6** < **3** < **4** < **5** (Table 2). Among them, the binding constant [$(2.7 \pm 0.3) \times 10^8 \text{ M}^{-1}$] of **5** with poly(dA)·2poly(dT) was the highest, almost 1000-fold more than that of neomycin.

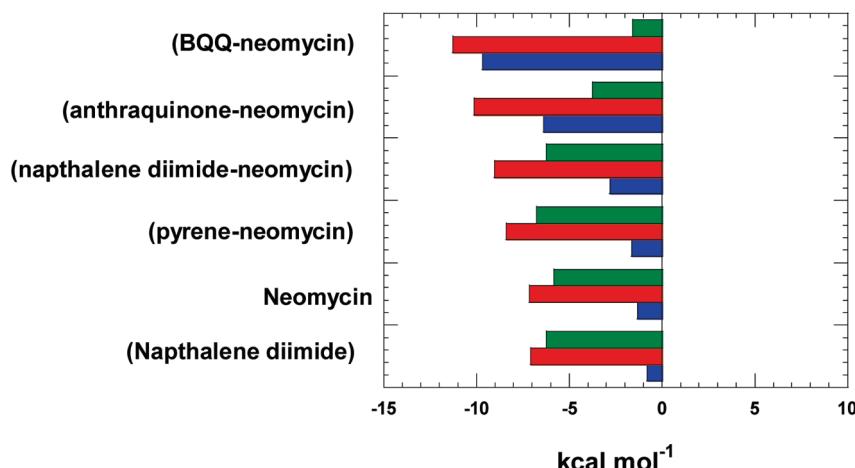


FIGURE 11. Thermodynamics of binding interactions of ligands with poly(dA)·2poly(dT) triplex at pH 5.5. Red bars represent ΔG , blue bars represent ΔH , and green bars represent $T\Delta S$. Experimental conditions: sodium cacodylate (10 mM), EDTA (0.5 mM), KCl (150 mM), $T = 20^\circ\text{C}$, pH 5.5. Reprinted with permission from ref 50. Copyright 2010 American Chemical Society.

The binding of compounds **3–6** with poly(dA)·2poly(dT) was mostly enthalpy-driven and gave negative ΔC_p values (Table 2). While discussing the small-molecule–DNA interactions, the negative sign of ΔC_p generally has been used to suggest removal of large amounts of nonpolar surface upon complex formation. ΔC_p values can however be impacted by a number of other factors including the release of constrained water molecules from the hydration shell, binding induced changes in internal vibrational modes, and electrostatic interactions. Surprisingly, most small molecules that bind to nucleic acids have been shown to have negative ΔC_p values⁵⁶ and aminoglycoside–nucleic-acid (RNA, DNA triplex, quadruplex) interactions universally show negative ΔC_p values.

These results provided a measure of quantification of triplex binding when the ligand surface area is increased in the order pyrene < naphthalenediimide < anthraquinone < BQQ. It is not a surprise that **5** is the most potent DNA triplex stabilizing agent among all the intercalator–neomycin conjugates in this study because the BQQ moiety (**5**) was developed by Hélène et al. as a rationally designed DNA triplex specific binding ligand.³⁷

Figure 11 shows the breakdown of thermodynamic properties of ligand–triplex interactions which have been discussed, in detail, in a recent report³⁶ and are summarized below. Neomycin is a major groove binder,⁴⁵ naphthalene diimide is a well-known intercalator,⁵⁷ and neomycin conjugates **3–6** bind through dual recognition mode (major groove and intercalation). (a) The binding of neomycin (groove binder) as well as the naphthalene diimide (intercalator) are largely driven by enthalpic contributions. (b) As

one goes from from pyrene–neomycin to BQQ–neomycin, an increase in intercalator surface area leads to an increase in the enthalpy of interaction, which clearly leads to a more negative free energy of binding. (c) The free energy $\Delta G_{\text{naphthalenediimide-neomycin:triplex}}$ is not a sum of free energy of $\Delta G_{\text{neomycin:triplex}}$ and $\Delta G_{\text{naphthalenediimide:triplex}}$, but leads to a $\Delta G_{\text{coupling}}$ of ~ 5 kcal/mol. While the $\Delta H_{\text{naphthalenediimide-neomycin:triplex}}$ increases substantially over $\Delta H_{\text{neomycin:triplex}}$, the corresponding increase in ΔS is not observed, suggesting that the conjugate, as linked, pays an entropic cost of covalently bridging the groove binder to the intercalator. (d) For the higher affinity conjugates, BQQ–neomycin, and anthraquinone–neomycin, the entropic contribution to $\Delta G_{\text{complexation}}$ drops substantially. Different linker length variations will lead to even better design of triplex-specific ligands and are currently being investigated. This approach utilizing dual recognition of DNA triplets should be widely applicable to development of structure-specific ligands for a host of nucleic acids where such groove binding and intercalating ligands with independent binding sites can be identified.

7. Conclusions and Future Directions

- A large number of DNA triplex intercalators (selective and nonselective) have been used to stabilize triplex structures, but the selective targeting of DNA/RNA triplex grooves has been difficult to achieve. Data presented here show that neomycin is unique in targeting nucleic acid triplex grooves. Molecular recognition of DNA triplex by neomycin offers a different recognition motif when compared to other known groove binders

- which overwhelmingly prefer the W–C duplex minor groove.
- b. RNA and hybrid duplexes and triplets are much harder to stabilize. DNA/RNA triplets only form at multimolar (2–4 M) salt concentrations. These findings show great promise for neomycin induced hybrid duplex and triplet stabilization under physiologically relevant salt conditions.
 - c. A number of X-ray and NMR structures are available for complexes of compounds that bind to both DNA and RNA by intercalation, and on compounds that bind in the DNA minor groove. Similar success has been attained in solving the structures of proteins and peptides that bind in the major groove of DNA and RNA. There is, however, little information available for small molecules that selectively bind DNA triplet grooves or RNA triplet grooves. In fact, no triplet X-ray structures have ever been solved (with the exception of some triplet duplex junctions). Recently, structures of drug–quadruplex have been solved.⁵⁸ It is the author's hope that ligand (neomycin)–triplet complexation will allow ligand–triplet X-ray structures to be solved in the near future, even though his personal efforts in crystallization of DNA triplet–neomycin complexes have been unsuccessful. NMR studies could also shed important light on details of aminoglycoside binding to the DNA triplet and are currently being pursued.
 - d. Repeating copolymers of polypurine and polypyrimidine sequences can assume a hinged DNA structure (H-DNA) which consists of triple-stranded and single-stranded regions. The H-DNA prone sequences are abundant in eukaryotes,^{59–61} suggesting that binding to the bacterial ribosome may not be the only site of action for these compounds and modified aminoglycosides that can permeate nucleus and mitochondria should be investigated for their binding to non-RNA A-form structures present in the cell. While aminoglycosides on their own do not internalize into eukaryotic cells,³⁸ conjugated aminoglycosides such as benzimidazole–neomycin, BQQ–neomycin, and antraquinone–neomycin rapidly internalize into DU145 cells as seen using confocal laser scanning microscopy (unpublished results). These findings have prompted the exploration of these conjugates in targeting Friedreich's ataxia (GAA)_n repeats and in triplet-forming sequences in malaria.^{21,62} Since the intercalator–aminosugar conjugates bind numerous nucleic acids (Figure 10), their benefits in targeting the small percent of TAT triplet-forming sites will require an assessment of their triplet binding specificity, but an equally important aspect that should also be explored is their permeability and localization in the cells. Conjugates that show preference for nuclear or mitochondrial localization in addition to triplet over duplex specificity would be promising candidates for triplet targeting, since their transport will allow them to bypass the large excess of RNA targets on the way to the DNA.
 - e. Data presented here, in addition to previous work, clearly suggest that aminoglycoside binding is not simply RNA selective but selective for nucleic acids that can adopt the A-conformation, such as RNA duplex, A-form DNA duplex, DNA/RNA hybrid duplex, DNA triplet, and RNA triplet.^{29,30,33,34,36,49,51,54,55,63–66} When studying new aminosugar modifications, it would therefore be important to know their effects on different nucleic acids to ascertain their selectivities.
 - f. These findings, in addition to the fact that neomycin also helps deliver ODNs to cells,⁶⁷ has led to the development of covalent aminoglycoside–ODN conjugates to target hybrid duplex- and triplet-forming sites in the mammalian and bacterial genome.^{51–53} Previous work in targeting the DNA duplex has included the use of intercalator, polyamine, and groove binder–ODN conjugates that have been shown to target multiple sites in the mammalian genome. These approaches have shown success in developing high affinity triplet-forming oligonucleotides (TFOs) that have been able to sequence-specifically recognize duplex DNA, to act as artificial nucleases, and to target a number of genes to regulate diseases such as HIV and cancer.^{68–71} Previous studies with TFOs linked to spermine,⁷² acridine,⁷³ Hoechst 33258,⁷⁴ cyclopropapyrroloindole,⁷⁵ and psoralen⁷⁶ have shown remarkable increases in triplet stabilization. However, their *selectivity* for triplet stabilization, as compared to duplex stabilization, is much lower than the selectivity of neomycin.³⁴ The duplex selectivity has limited intercalator conjugation to the end of the TFOs where intercalation is restricted to duplex–triplet junctions.⁷⁷ Our approach, where neomycin can be coupled every five to six triplets,⁵¹ is thus unattainable with intercalator conjugates. Alternative and unpredictable mechanisms of gene repression where the intercalating

agent might induce bending or distortion of the DNA, alter chromatin structure, or recruit DNA topoisomerase II and DNA repair enzymes are a concern. The intercalator's lack of specificity has also been noted¹⁵ in potential clinical applications of TFO. For this reason, intercalators have largely been restricted to one attachment per ODN, so that the conjugate retains the binding properties of the ODN and not that of the intercalator.

Improvements in (a) ODN binding to the duplex and (b) ODN transfection are needed to allow ODN directed triple helix therapies to advance to the clinic. Previous approaches in TFO–small-molecule conjugates have been designed to improve only one of the two limitations mentioned above. Neomycin considerably enhances the binding affinities of TFOs to their duplex DNA target (*vide infra*). Additionally, neomycin, when combined with a cationic lipid preparation, such as DOTAP, enhances transfection efficiency of both reporter plasmids and ODNs in cancer cells.⁶⁷ As more aminoglycoside-specific triplex and duplex sequences are identified, highly specific ODN–aminoglycoside conjugates can then be developed for targeting DNA and RNA, leading to novel approaches in antisense or antigene therapies.

This study was made possible by funds from NSF (CHE/MCB-0134932) and NIH (R15CA125724). I am thankful to the numerous students and postdoctoral fellows who contributed to this work.

BIOGRAPHICAL INFORMATION

Dev P. Arya is an Associate Professor of Chemistry in the Palmetto state at Clemson University, Clemson, SC. He received his B.S. with Honors in chemistry from St. Stephen's College, Delhi, India. His primary research interests lie in the area of nucleic acid recognition.

FOOTNOTES

*Telephone: 1-864-656-1106. Fax: 1-864-656-6613. E-mail: dparya@clemson.edu.

REFERENCES

- Kool, E. T. Design of Triplex-forming Oligonucleotides for Binding DNA and RNA: Optimizing Affinity and Selectivity. *New J. Chem.* **1997**, *21*, 33–45.
- Dervan, P. B. Molecular Recognition of DNA by Small Molecules. *Bioorg. Med. Chem.* **2001**, *9*, 2215–2235.
- Felsenfeld, G.; Davies, D.; Rich, A. Formation of a Three-Stranded Polynucleotide Molecule. *J. Am. Chem. Soc.* **1957**, *79*, 2023–2024.
- Felsenfeld, G.; Rich, A. Studies on the Formation of Two and Three Stranded Polyribonucleotides. *Biochim. Biophys. Acta* **1957**, *26*, 457–468.
- Maher, L. J., III; Wold, B.; Dervan, P. B. Inhibition of DNA Binding Proteins by Oligonucleotide-Directed Triple Helix Formation. *Science* **1989**, *245*, 725–730.
- Moser, H. E.; Dervan, P. B. Sequence-Specific Cleavage of Double Helical DNA by Triple Helix Formation. *Science* **1987**, *238*, 645–650.
- Beal, P. A.; Dervan, P. B. Second structural motif for recognition of DNA by oligonucleotide-directed triple-helix formation. *Science* **1991**, *251*, 1360–1363.
- Giovannangeli, C.; Montenay-Garestier, T.; Rougee, M.; Chassignol, M.; Thuong, N. T.; Hélène, C. Single-Stranded DNA as a Target for Triple-Helix Formation. *J. Am. Chem. Soc.* **1991**, *113*, 7775–7777.
- Hélène, C.; Toulme, J. J. Specific Regulation of Gene Expression by Antisense, Sense, and Antigene Nucleic Acids. *Biochim. Biophys. Acta* **1990**, *1049*, 99–125.
- Kool, E. T. Recognition of DNA, RNA, and Proteins by Circular Oligonucleotides. *Acc. Chem. Res.* **1998**, *31*, 502–510.
- Duca, M.; Vekhoff, P.; Oussedik, K.; Halby, L.; Arimondo, P. B. The triple helix: 50 years later, the outcome. *Nucleic Acids Res.* **2008**, *36*, 5123–5138.
- Schleifman, E. B.; Chin, J. Y.; Glazer, P. M. Triplex-mediated gene modification. *Methods Mol. Biol.* **2008**, *435*, 175–190.
- Jain, A.; Wang, G.; Vasquez, K. M. DNA triple helices: biological consequences and therapeutic potential. *Biochimie* **2008**, *90*, 1117–1130.
- Jain, A.; Magistri, M.; Napoli, S.; Carbone, G. M.; Catapano, C. V. Mechanisms of triplex DNA-mediated inhibition of transcription initiation in cells. *Biochimie* **2010**, *92*(3), 317–320.
- Seidman, M. M.; Glazer, P. M. The potential for gene repair via triple helix formation. *J. Clin. Invest.* **2003**, *112*, 487–494.
- Vazquez, K. M.; Glazer, P. M. Triplex Forming Oligonucleotides: principles and applications. *Q. Rev. Biophys.* **2002**, *35*, 89–107.
- Lestienne, P. P. Are there three polynucleotide strands in the catalytic centre of DNA polymerases? *Biochimie* **2009**, *97*, 1523–1530.
- Strekowski, L.; Hojat, M.; Wolinska, E.; Parker, A. N.; Paliakov, E.; Gorecki, T.; Tanious, F. A.; Wilson, W. D. New triple-helix DNA stabilizing agents. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 1097–1100.
- Holt, P. A.; Ragazzon, P.; Strekowski, L.; Chaires, J. B.; Trent, J. O. Discovery of novel triple helical DNA intercalators by an integrated virtual and actual screening platform. *Nucleic Acids Res.* **2009**, *37*, 1280–1287.
- Chaires, J. B.; Ren, J.; Henary, M.; Zegrocka, O.; Bishop, G. R.; Strekowski, L. Triplex selective 2-(2-naphthyl)quinoline compounds: origins of affinity and new design principles. *J. Am. Chem. Soc.* **2003**, *125*, 7272–7283.
- Bergquist, H.; Nikravesh, A.; Fernandez, R. D.; Larsson, V.; Nguyen, C. H.; Good, L.; Zain, R. Structure-specific recognition of Friedreich's ataxia (GAA)n repeats by benzoquinolinoquinoxaline derivatives. *ChemBioChem* **2009**, *10*, 2629–2637.
- Lane, A.; Jenkins, T. Structures and properties of multi-stranded nucleic acids. *Curr. Org. Chem.* **2001**, *5*, 845–869.
- Antony, T.; Thomas, T.; Shirahata, A.; Sigal, L. H.; Thomas, T. J. Selectivity of Spermine Homologs on Triplex DNA Stabilization. *Antisense Nucleic Acid Drug Dev.* **1999**, *9*, 221–231.
- Neidle, S. DNA minor-groove recognition by small molecules. *Nat. Prod. Rep.* **2001**, *18*, 291–309.
- Dervan, P. B.; Edelson, B. S. Recognition of the DNA minor groove by pyrrole-imidazole polyamides. *Curr. Opin. Struct. Biol.* **2003**, *13*, 284–299.
- Kopka, M. L.; Yoon, C.; Goodsell, D.; Pjura, P.; Dickerson, R. E. The Molecular Origin of DNA Drug Specificity in Netropsin and Distamycin. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 1376–1380.
- Dervan, P. B. Design of Sequence-Specific DNA-Binding Molecules. *Science* **1986**, *232*, 464–71.
- Wartell, R. M.; Larson, J. E.; Wells, R. D. Netropsin. A specific probe for A-T regions of duplex deoxyribonucleic acid. *J. Biol. Chem.* **1974**, *249*, 6719–6731.
- Willis, B.; Arya, D. P. An Expanding View of Aminoglycoside-Nucleic Acid Recognition. In *Advances in Carbohydrate Chemistry and Biochemistry*; Horton, D., Ed.; Elsevier: Washington, D.C., 2006; Vol. 60, pp 251–302.
- Arya, D. P. Aminoglycoside-nucleic acid interactions: the case for neomycin. *Top. Curr. Chem.* **2005**, *253*, 149–178.
- Waksman, S. A.; Lechevalier, H. A. Neomycin, a New Antibiotic Active against Streptomycin-Resistant bacteria, Including Tuberculosis Organisms. *J. Antibiot.* **1949**, *109*, 305–309.
- Schatz, A.; Bugie, E.; Waksman, S. A. Streptomycin, a substance exhibiting antibiotic activity against Gram-positive and Gram-negative bacteria. *Proc. Soc. Exp. Biol. Med.* **1944**, *55*, 66–69.
- Arya, D. P.; Coffee, R. L. DNA triple helix stabilization by aminoglycoside antibiotics. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 1897–1899.
- Arya, D. P.; Coffee, R. L.; Willis, B.; Abramovitch, A. I. Aminoglycoside-Nucleic Acid Interactions: Remarkable Stabilization of DNA and RNA Triple Helices by Neomycin. *J. Am. Chem. Soc.* **2001**, *123*, 5385–5395.
- Xi, H.; Kumar, S.; Dosen-Micovic, L.; Arya, D. P. Calorimetric and spectroscopic studies of aminoglycoside binding to AT-rich DNA triple helices. *Biochimie* **2010**, *92*, 514–529.
- Arya, D. P.; Xue, L.; Tenant, P. Combining the Best in Triplex Recognition: Synthesis and Nucleic Acid Binding of a BQQ-Neomycin Conjugate. *J. Am. Chem. Soc.* **2003**, *125*, 8070–8071.

- 37 Zain, R.; Marchand, C.; Sun, J.; Nguyen, C. H.; Bisagni, E.; Garestier, T.; Hélène, C. Design of a Triple-Helix-Specific Cleaving Reagent. *Chem. Biol.* **1999**, *6*, 771–777.
- 38 Luedtke, N. W.; Carmichael, P.; Tor, Y. Cellular uptake of aminoglycosides, guanidinoglycosides, and poly-arginine. *J. Am. Chem. Soc.* **2003**, *125*, 12374–12375.
- 39 Radhakrishnan, I.; Patel, D. J. DNA Tripleplexes: Solution Structures, Hydration Sites, Energetics, Interactions, and Function. *Biochemistry* **1994**, *33*, 11405–11416.
- 40 Radhakrishnan, I.; Patel, D. J. Solution structure of a purine.purine.pyrimidine DNA triplex containing G.GC and T.AT triples. *Structure* **1993**, *1*, 135–152.
- 41 Shields, G. C.; Laughton, C. A.; Orozco, M. Molecular dynamics simulations of the d(T.A.T) Triple Helix. *J. Am. Chem. Soc.* **1997**, *119*, 7463–7469.
- 42 Feigon, J.; Wang, E. Structures of nucleic acid tripleplexes. In *Nucleic Acid Structure*; Neidle, S., Ed.; Oxford University Press: Oxford, 1999; Vol. 1, pp 355–388.
- 43 Phipps, A. K.; Tarkoy, M.; Schultze, P.; Feigon, J. Solution Structure of an Intramolecular DNA Triplex Containing 5-(1-propynyl)-2'-deoxyuridine Residues in the Third Strand. *Biochemistry* **1998**, *37*, 5820–5830.
- 44 Tarkoy, M.; Phipps, A. K.; Schultze, P.; Feigon, J. Solution Structure of an Intramolecular DNA Triplex Linked by Hexakis(ethylene glycol) Units: d(AGAGAGAA-(EG)₆-TTCTCT-(EG)₆TCTCTT). *Biochemistry* **1998**, *37*, 5810–5819.
- 45 Arya, D. P.; Micovic, L.; Charles, I.; Coffee, R. L.; Willis, B.; Xue, L. Neomycin Binding to DNA Triplex Watson-Hoogsteen (W-H) Groove: A Model. *J. Am. Chem. Soc.* **2003**, *125*, 3733–3744.
- 46 Kool, E. T. Preorganization of DNA: Design Principles for Improving Nucleic Acid Recognition by Synthetic Oligonucleotides. *Chem. Rev.* **1997**, *97*, 1473–1487.
- 47 Kool, E. T. Circular oligonucleotides: new concepts in oligonucleotide design. *Annu. Rev. Biophys. Biomol. Struct.* **1996**, *25*, 1–28.
- 48 Prakash, G.; Kool, E. T. Molecular Recognition by Circular Oligonucleotides. Strong Binding of single-stranded DNA and RNA. *Chem. Commun.* **1991**, *116*, 1161–1163.
- 49 Arya, D. P.; Coffee, R. L.; Charles, I. Neomycin-Induced Hybrid Triplex Formation. *J. Am. Chem. Soc.* **2001**, *123*, 11093–11094.
- 50 Xue, L.; Xi, H.; Kumar, S.; Gray, D.; Davis, E.; Hamilton, P.; Skriba, M.; Arya, D. P. Probing the recognition surface of a DNA triplex: Binding studies with intercalator-neomycin conjugates. *Biochemistry* **2010**, *49*, 5540–5552.
- 51 Charles, I.; Xi, H.; Arya, D. P. Sequence-specific targeting of RNA with a neomycin-DNA conjugate. *Biconjugate Chem.* **2007**, *18*, 160–169.
- 52 Charles, I.; Arya, D. P. Synthesis of Neomycin-DNA/Peptide Nucleic Acid Conjugates. *J. Carbohydr. Chem.* **2005**, *24*, 143–157.
- 53 Charles, I.; Xue, L.; Arya, D. P. Synthesis of Aminoglycoside-DNA Conjugates. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 1259–1262.
- 54 Shaw, N. N.; Xi, H.; Arya, D. P. Molecular recognition of a DNA:RNA hybrid: sub-nanomolar binding by a neomycin-methidium conjugate. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 4142–4145.
- 55 Arya, D. P.; Xue, L.; Willis, B. Aminoglycoside (neomycin) preference is for A-form nucleic acids, not just RNA: Results from a competition dialysis study. *J. Am. Chem. Soc.* **2003**, *125*, 10148–10149.
- 56 Chaires, J. B. Energetics of Drug-DNA Interactions. *Biopolymers* **1998**, *44*, 201–215.
- 57 Gianollo, D. A.; Segismundo, J. M.; McLaughlin, L. W. Tethered naphthalene diimide-based intercalators for DNA triplex stabilization. *Nucleic Acids Res.* **2000**, *28*, 2128–2134.
- 58 Neidle, S. The structures of quadruplex nucleic acids and their drug complexes. *Curr. Opin. Struct. Biol.* **2009**, *19*, 239–250.
- 59 Schroth, G. P.; Ho, P. S. Occurrence of potential cruciform and H-DNA forming sequences in genomic DNA. *Nucleic Acids Res.* **1995**, *23*, 1977–1983.
- 60 Mirkin, S. M.; Frank-Kamenetskii, M. D. H-DNA and Related Structures. *Annu. Rev. Biophys. Biomol. Struct.* **1994**, *23*, 541–576.
- 61 Htun, H.; Dahlberg, J. E. Topology and Formation of Triple-Stranded H-DNA. *Science* **1989**, *243*, 1571–1576.
- 62 Horrocks, P.; Wong, E.; Russell, K.; Emes, R. D. Control of gene expression in Plasmodium falciparum - ten years on. *Mol. Biochem. Parasitol.* **2009**, *164*, 9–25.
- 63 Xi, H.; Gray, D.; Kumar, S.; Arya, D. P. Molecular recognition of single-stranded RNA: neomycin binding to poly(A). *FEBS Lett.* **2009**, *583*, 2269–2275.
- 64 Shaw, N. N.; Arya, D. P. Recognition of the unique structure of DNA:RNA hybrids. *Biochimie* **2008**, *90*, 1026–1039.
- 65 Xi, H.; Arya, D. P. Recognition of triple helical nucleic acids by aminoglycosides. *Curr. Med. Chem.: Anti-Cancer Agents* **2005**, *5*, 327–338.
- 66 Xue, L.; Charles, I.; Arya, D. P. Pyrene-neomycin conjugate: dual recognition of a DNA triple helix. *Chem. Commun.* **2002**, *1*, 70–71.
- 67 Napoli, S.; Carbone, G. M.; Catapano, C.; Shaw, N.; Arya, D. P. Neomycin improves cationic lipid-mediated transfection of DNA in human cells. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 3467–3469.
- 68 Benfield, A. P.; Macleod, M. C.; Liu, Y.; Wu, Q.; Wensel, T. G.; Vasquez, K. M. Targeted generation of DNA strand breaks using pyrene-conjugated triplex-forming oligonucleotides. *Biochemistry* **2008**, *47*, 6279–6288.
- 69 Oh, D. H.; Hanawalt, P. C. Triple helix-forming oligonucleotides target psoralen adducts to specific chromosomal sequences in human cells. *Nucleic Acids Res.* **1999**, *27*, 4734–4742.
- 70 Giovannangeli, C.; Perrouault, L.; Escude, C.; Thuong, N.; Hélène, C. Specific Inhibition of in Vitro Transcription Elongation by Triplex-Forming Oligonucleotide-Intercalator Conjugates Targeted to HIV Proviral DNA. *Biochemistry* **1996**, *35*, 10539–10548.
- 71 Francois, J.; Saison-Behmoaras, T.; Barbier, C.; Chassignol, M.; Thuong, N. T.; Hélène, C. Sequence-specific recognition and cleavage of duplex DNA via triple-helix formation by oligonucleotides covalently linked to a phenanthroline-copper chelate. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 9702–9706.
- 72 Rajeev, K. G.; Jadhav, V. R.; Ganesh, K. N. Triplex formation at physiological pH: comparative studies on DNA tripleplexes containing 5-Me-dC tethered at N4 with spermine and tetraethyleneoxyamine. *Nucleic Acids Res.* **1997**, *25*, 4187–4193.
- 73 Stonehouse, T. J.; Fox, K. R. DNase I footprinting of triple helix formation at polypurine tracts by acridine-linked oligopyrimidines: stringency, structural changes and interaction with minor groove binding ligands. *Biochim. Biophys. Acta, Gene Struct. Expression* **1994**, *1218*, 322–330.
- 74 Robles, J.; McLaughlin, L. W. DNA Triplex Stabilization Using a Tethered Minor Groove Binding Hoechst 33258 Analogue. *J. Am. Chem. Soc.* **1997**, *119*, 6014–6021.
- 75 Dempcy, R. O.; Kutyavin, I. V.; Mills, A. G.; Lukhtanov, E. A.; Meyer, R. B. Linkers designed to intercalate the double helix greatly facilitate DNA alkylation by triplex-forming oligonucleotides carrying a cyclopropapyrrolindole reactive moiety. *Nucleic Acids Res.* **1999**, *27*, 2931–2937.
- 76 Liu, Y.; Nairn, R. S.; Vasquez, K. M. Targeted gene conversion induced by triplex-directed psoralen interstrand crosslinks in mammalian cells. *Nucleic Acids Res.* **2009**, *37*, 6378–6388.
- 77 Zhou, B.; Puga, E.; Sun, J.; Garestier, T.; Hélène, C. Stable Triple Helices Formed by Acridine-Containing Oligonucleotides with Oligopurine Tracts of DNA Interrupted by One or Two Pyrimidines. *J. Am. Chem. Soc.* **1995**, *117*, 10425–10428.