

Sequence-Specific Targeting of RNA with an Oligonucleotide–Neomycin Conjugate

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The synthesis of neomycin covalently attached at the C5-position of 2'-deoxyuridine is reported. The synthesis outlined allows for incorporation of an aminoglycoside (neomycin) at any given site in an oligonucleotide (ODN) where a thymidine (or uridine) is present. Incorporation of this modified base into an oligonucleotide, which is complementary to a seven-bases-long α -sarcin loop RNA sequence, leads to enhanced duplex hybridization. The increase in T_m for this duplex ($\Delta T_m = 6$ °C) suggests a favorable interaction of neomycin within the duplex groove. CD spectroscopy shows that the modified duplex adopts an A-type conformation. ITC measurements indicate the additive effects of ODN and neomycin binding to the RNA target ($K_a = 4.5 \times 10^7$ M⁻¹). The enhanced stability of the hybrid duplex from this neomycin–ODN conjugate originates primarily from the enthalpic contribution of neomycin [$\Delta\Delta H_{\text{obs}} = -7.21$ kcal/mol ($\Delta H_{\text{neomycin conjugated}} - \Delta H_{\text{nonconjugated}}$)] binding to the hybrid duplex. The short linker length allows for selective stabilization of the hybrid duplex over the hybrid triplex. The results described here open up new avenues in the design and synthesis of nucleo–aminoglycoside–conjugates (N–Ag–C) where the inclusion of any number of aminoglycoside (neomycin) molecules per oligonucleotide can be accomplished.

INTRODUCTION

Aminoglycoside antibiotics interact with a variety of RNA molecules and exhibit bactericidal activity by inducing the misreading of codons (1, 2) as well as inhibition of translocation (3). A number of RNA molecules have been known to interact with aminoglycosides; group I introns (4, 5), ribonuclease P RNA (6), hairpin ribozyme (7, 8), hammerhead ribozyme (9–11), hepatitis delta virus ribozyme (12, 13), untranslated region of thymidylate synthase mRNA (14), hTR RNA component (15), human mRNAs and both reverse response element and transactivating response element RNA motifs of HIV-1 (16–19). We have identified neomycin (Figure 1) as the most potent DNA/RNA/hybrid triplex stabilizing groove binder (20, 21). Neomycin, an aminoglycoside, had largely been known to bind different RNA structures (10, 14, 22–24). Recent studies from our laboratory have, however, revealed that aminoglycosides (in particular, neomycin) can bind to other A-form structures (25). The stabilization of the poly(dA)•2poly(dT) (20), small triplexes (26), DNA•RNA hybrid duplexes, RNA triplex, and hybrid triple helices (27) by neomycin has recently been reported by us. Aminoglycosides most likely bind in the major groove of these structures (much like RNA, as the A-form nucleic acids have a narrower major groove) (25, 28).

Carbohydrate (Neomycin)–DNA Conjugates. In contrast to abundant glycosylated protein and lipids in nature, few examples of glycosylated nucleic acids have been reported. A few reports discuss glycosylated nucleosides (29–31) and their role in gene expression (32, 33). The presence of these glycosylated nucleosides modulates the functions of *Escherichia coli* bacteriophages of the T-even series (34), genome of *Trypanosoma brucei* (a protozoan found in phages and known to cause African sleeping sickness) (35), and *Diplonema* (a small phagotrophic marine flagellate) (36). These reports in carbohydrate–nucleic acid

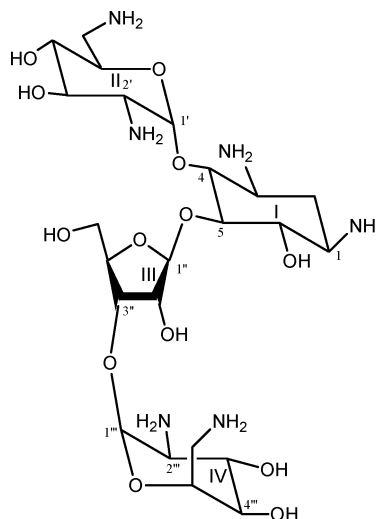


Figure 1. Structure of neomycin.

interactions have also ignited interest in the development of such conjugates. The importance of aminoglycosides in the recognition of nucleic acids prompted us to design, synthesize, and evaluate aminoglycoside–oligonucleotide conjugates. If carbohydrates (aminoglycosides) can be conjugated to DNA without affecting the structure and stability of DNA, and bind to their nucleic acid target (RNA) with specificity, then these conjugates will be of interest as a new class of artificial glycoconjugate materials applicable to cell-targeted gene therapy techniques. A few examples are also known in which carbohydrate ligands have been used to increase the uptake of antisense oligonucleotides (38). These examples include the use of mannosylated polylysine (39) and galactosylated polylysine (40) in receptor-mediated endocytosis of antisense oligonucleotides. We have recently shown that neomycin assists in the lipid-mediated delivery of plasmid DNA and oligonucleotides (41). Delivery of oligonucleotides has been a major impediment in the

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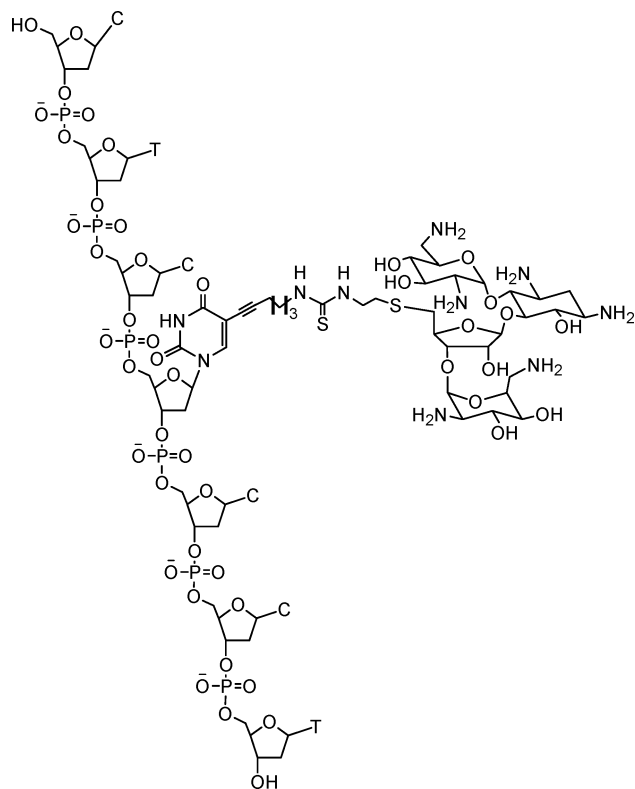


Figure 2. Structure of the 7mer–neo conjugate.

development of all nucleic acid based drugs. We recently showed that neomycin, an aminoglycoside antibiotic, when combined with a cationic lipid preparation, like DOTAP, enhances transfection efficiency of both reporter plasmids and oligonucleotides and results in a significant increase in transgene expression (41).

To take advantage of these unique findings of aminoglycoside–nucleic acid interaction, we have initiated the development of aminoglycoside–nucleic acid conjugates. Recently, we reported synthesis of neomycin conjugates in which neomycin was conjugated at the 5'-end of the nucleic acid analogs (DNA/PNA) via thiourea linkage (42, 43). The application of such conjugates, however, is limited, as the drug can be added only at the 5'-end of the oligomer. The applicability of conjugating a drug molecule to any given site in an oligonucleotide can be accomplished if the drug is attached to the base or sugar using an appropriate linker, leaving 5'- and 3'-ends free for suitable oligomer elongation. With this methodology, the addition of drug–phosphoramidite conjugates to the growing strand during oligonucleotide synthesis can be monitored using trityl monitor. Herein, we describe an efficient phosphoramidite-based method for the synthesis of neomycin attached to the C5-position of 2'-deoxyuridine through a hexynyl linker, its extension to oligonucleotide–neomycin synthesis (Figure 2), and its hybridization with a complementary RNA structure. These conjugates will be referred to as nucleo–aminoglycoside conjugates (N–Ag–C), of which the first example, nucleo–neomycin conjugate (N–Neo–C), is reported here.

Selection of Target RNA. Among functionally active 16S rRNA target sites, A-site (1, 44–47) and α -sarcin loop (47–50) are important, as they have a profound effect on protein synthesis. The α -sarcin loop is the region where α -sarcin catalyzes the hydrolysis of the phosphodiester bond that links its eighth and ninth residues (50). This causes improper binding of ribosomes to elongation factors, which eventually terminates protein synthesis (47). The importance of this single phosphodiester linkage emphasizes the significance of the α -sarcin loop

as an attractive target for antisense oligonucleotides. We therefore chose this target for our initial studies.

EXPERIMENTAL PROCEDURES

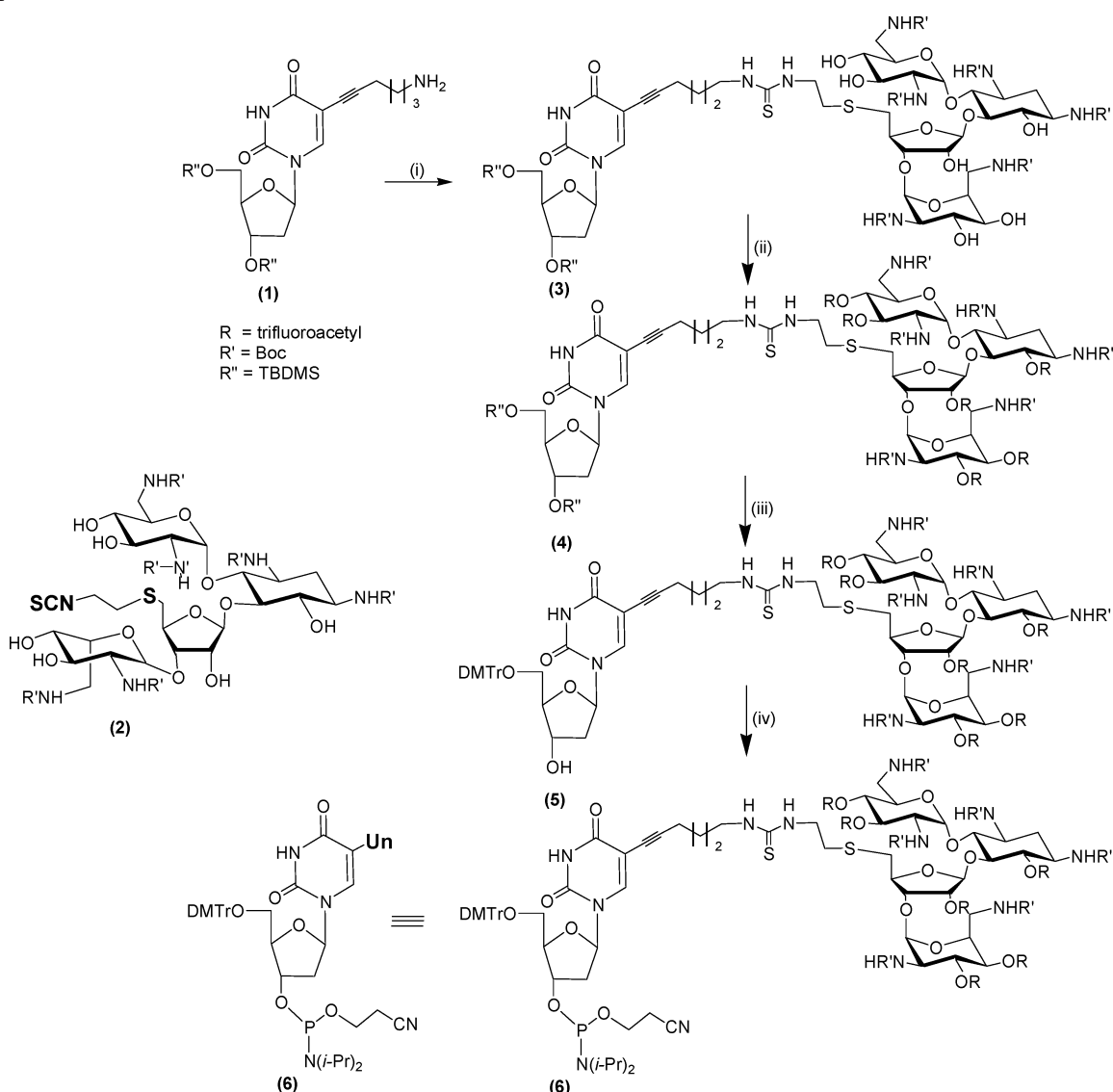
Materials. All commercial reagents were used without further purification. Pyridine, methylene chloride, and *N,N*-dimethylformamide (DMF) were refluxed over calcium hydride and distilled. All reactions were carried out in oven-dried glassware under N_2 /argon atmosphere. All reactions were monitored by thin-layer chromatography (TLC) with precoated silica gel on glass plate. Visualization of the TLC plate was achieved with either UV light or spraying ethanolic ninhydrin solution followed by heating the plate with a heat gun. ICN silica gel 32–63 (60 Å) was used for column chromatography. 1H NMR spectra were recorded by using either 300 MHz or 500 MHz NMR. IR was recorded on a Nicolet Magna-IR Spectrometer 550 as a solution of either 1,2-dichloroethane or CCl_4 , and the peaks corresponding to the solvent was subtracted manually. MALDI-TOF mass was recorded on a Bruker Daltonics OmniFLEX Bench-Top MALDI-TOF mass spectrometer. Preparative anion exchange HPLC was carried out on a Phenomenex SAX 80 Å column (10 × 250 mm, 5 μ), and for analytical anion exchange HPLC, a Waters Gen-Pak FAX (4.6 × 100 mm) column was used. Preparative and analytical RP-HPLC were carried out with Alltima C8 100 Å (250 × 4.6 mm, 10 μ) column.

Please see Supporting Information for experimental procedures for synthesis of monomers. 7mer DNAs (dY, N-dY, and dY') were synthesized on Expedite Nucleic Acid System (8909) and purified by HPLC. RNA (rR) was purchased from Dharmacon, Inc., (Lafayette, CO) and was used without further purification (lot numbers COFRA-0001 and CHAIB-0001). The concentrations of all the nucleic acid solutions were determined spectrophotometrically using the following extinction coefficients (on a per strand basis, $M^{-1} cm^{-1}$): $e_{260} = 81\,900$ for rR, and $e_{260} = 54\,000$ for dY. Neomycin (lot 129H0918) was purchased from Sigma (St. Louis, MO) and was used without further purification.

UV Spectroscopy. UV spectra were recorded at $\lambda = 200$ –300 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 accurate T_m determinations, first-derivative functions were used. Data were recorded every 1.0 °C. In all UV experiments, the samples were heated from 20 to 95 °C at a rate of 5 °C/min, annealed (95 to 5 °C, at a rate of 0.2 °C/min), and again melted (5 to 90 °C, at a rate of 0.2 °C/min), and the samples were brought back to 25 °C at a rate of 5 °C/min. During melting and annealing, the absorbance of each solution was monitored at the following wavelengths: 260, 280, and 284 nm. The second melting stage was used for calculating melting temperature.

CD Spectroscopy. All CD experiments were conducted at 15 °C on a JASCO J-810 spectropolarimeter equipped with a thermoelectrically controlled cell holder. A quartz cell with a 1 cm path length was used in the CD studies. CD spectra were recorded as an average of 3 scans from 220 to 320 nm with data recorded in 1 nm increments with an averaging time of 2 s. The hybrid duplex solution was incubated at 4 °C for 16 h before CD titration. After each addition of 5 μ L of neomycin (1 mM) to a 2 mL solution of hybrid duplex (10 μ M per duplex), the solution was inverted 3 times to ensure thorough mixing and incubated for 3 min prior to scanning. Buffer conditions for CD titration are as follows: 10 mM sodium cacodylate, 0.5 mM EDTA, 60 mM total Na^+ , and pH 6.0.

ITC Measurements. Isothermal titration calorimetric measurements were performed at 10 °C on a MicroCal VP-ITC (MicroCal, Inc.; Northampton, MA). In a typical experiment, 5

Scheme 1^a

^a Reagents and conditions: (i) (a) Neo-S-CH₂CH₂SCN, DMAP, pyridine (76%); (ii) (CF₃CO₂)O, *i*-Pr₂EtN, CH₂Cl₂ (75%); (iii) TBAF, DMF; and then DMTrCl, pyridine, DMAP (78%); (iv) NCCH₂CH₂OP[N(*i*-Pr)₂]₂, bis(diisopropylammonium) tetrazolide and CH₂Cl₂ (89%).

μL aliquots of 200 μM single strand were injected from a rotating syringe (300 rpm) into an isothermal sample chamber containing 1.42 mL of a RNA solution that was 20 μM per strand. Each experiment was accompanied by control experiments in which 5 μL aliquots of the single strands were titrated into buffer. The duration of each injection was 5.0 s, and the delay between each injection was 300 s. The initial delay prior to the first injection of 2 μL was 60 s. Each injection generated a heat burst curve (microcalories per second vs seconds). The area under each curve was determined by integration using the *Origin 5.0* software (MicroCal, Inc.; Northampton, MA) to obtain a measure of the heat associated with that injection. The buffer used in the experiment was 10 mM sodium cacodylate, 0.5 mM EDTA, 60 mM total Na⁺, and pH 7.0.

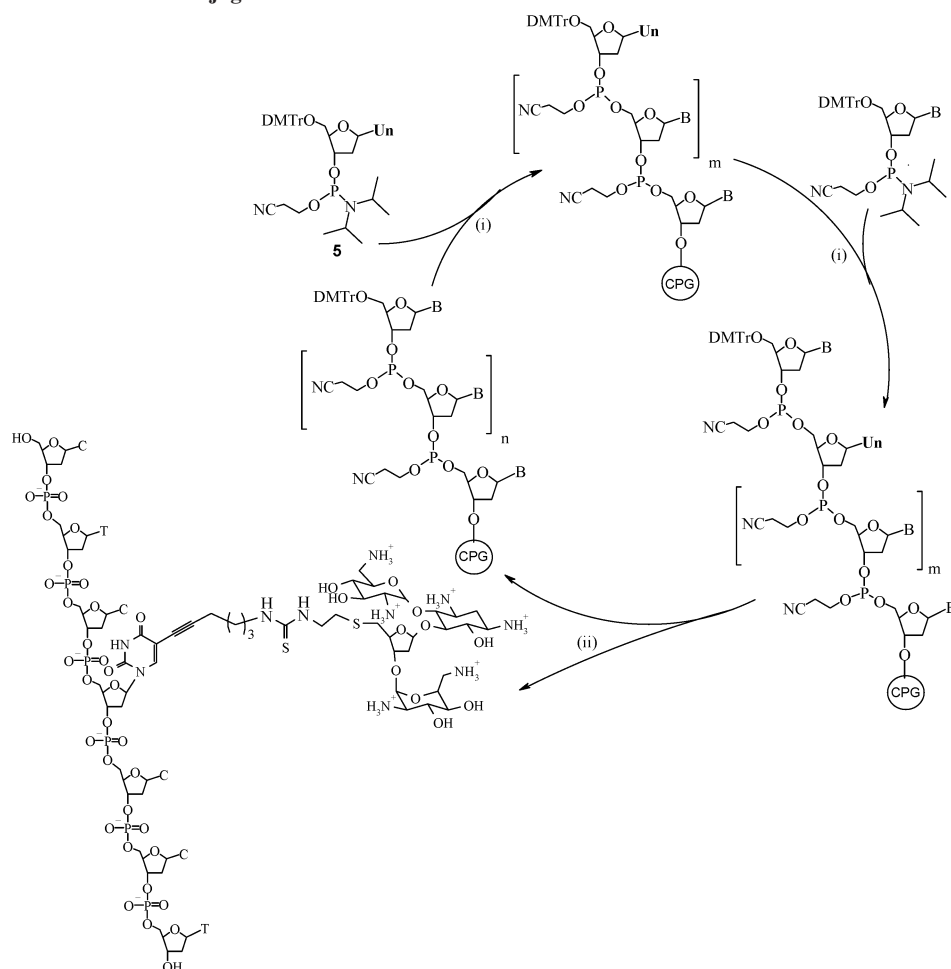
Covalent Attachment of Neomycin to an Oligonucleotide.

The oligonucleotide synthesis (1 μM) was carried out on Expedite Nucleic Acid Synthesis System (8909) using standard phosphoramidite chemistry (51). The coupling of deoxyuridine-neomycin conjugate 6 (0.2 M) to the oligomer was carried out for 30 min. After the oligomer synthesis, the CPG column was dried using argon gas. Then, the conjugate was detached from the solid support using NH₄OH, and the resulting solution was evaporated. The lyophilized sample was treated with 1 mL of

1,4-dioxane solution containing 3% CF₃CO₂H and 1% *m*-cresol (v/v/v %). After 30 min, the conjugate (Scheme 2) was precipitated with 10 mL of diethyl ether and the precipitate was washed with 3 \times 10 mL of diethyl ether. The solution was evaporated, and the residue was resuspended in triethylammonium acetate buffer 10 mM and extracted twice with ether. Ether was removed, and the aqueous layer was dried under vacuum for 1 h. The solid was redissolved in 1 mL of water, purified by anion-exchange chromatography, and lyophilized.

RESULTS AND DISCUSSION

Design. Our design is based on a computer model of neomycin bound to the hybrid duplex major groove, shown in Figure 3. There are two ways of approaching the major groove: either through a 5'-OH conjugation or through conjugation with a base. Only one neomycin can be conjugated through 5'-OH conjugation, which left the 5-uridine position as the desirable site for conjugation of multiple neomycins. The linker length (12 atoms between 5-uridine and neomycin 5'-CH₂-) chosen by us allows enough flexibility for neomycin to fit into the major groove and maintain the favorable contacts. Our previous results show that the amino groups on rings I, II, and

Scheme 2. Synthesis of 7mer-Neo Conjugate N-Neo-C^a

^a (i) Deprotection with 4% trichloroacetic acid in CH₂Cl₂, and coupling with **3** in the presence of 1*H*-tetrazole followed by capping with acetic anhydride in pyridine/THF solution, and oxidation with I₂ in THF/H₂O/pyridine solution; (ii) β -elimination followed by deprotection from the solid support using conc. NH₄OH; (c) 1,4-dioxane solution containing 3% CF₃CO₂H and 1% *m*-cresol (v/v/v %).

IV (Figure 1) of neomycin are necessary in stabilizing and recognizing various nucleic acid forms (aminoglycosides without any of these amines do not stabilize A-form structures as efficiently) (20).

Synthesis. On the basis of prior results mentioned above, the 5'-OH on ring III of neomycin was modified to neomycin isothiocyanate **2** as described in our earlier report (42). The precursor **1** was prepared from 2'-deoxyuridine by following a literature procedure (52). Then, neomycin isothiocyanate **2** was coupled with hexynylamino group of the modified 2'-deoxyuridine **1** to give conjugate **3** with an isothiourea linkage (Scheme 1). The hydroxyl groups on the neomycin molecule need to be protected in order to use phosphoramidite chemistry. The literature survey shows that the hydroxyl groups on aminoglycosides can be protected with an acetyl group (53). Deacylation can be carried out at the end of the oligonucleotide-conjugate synthesis with aqueous ammonia treatment, which can also perform the following in a single step: cleavage of the oligonucleotide from the solid support and β -elimination. Thus, the hydroxyl groups on neomycin were protected with a trifluoroacetyl group to get acetylated neomycin **4** in 75% yields. Deprotection of *t*-butyl dimethylsilyl (TBDMS) groups on the sugar with tetrabutylammonium fluoride (TBAF) followed by protection of 5'-hydroxyl group with DMTr-Cl in the presence of DMAP in pyridine gave the required TBDMS-free compound **5** in 78% yield. Further reaction of compound **5** with 2-cyanoethyl-*N,N,N',N'*-tetraisopropyl phosphoramidite in the presence of bis-(diisopropylammonium) tetrazolidine gave phosphoramidite

6 in good yields (54). For synthesis and characterization of compounds **1–6**, please see Supporting Information.

After synthesizing phosphoramidite **6**, the 7mer oligonucleotide (Figure 1) was prepared on a CPG column using standard phosphoramidite synthesis protocols (Scheme 2) (51). After introducing three bases, the modified base was coupled with the oligonucleotide on the solid support for 30 min, which was followed by the addition of the remaining three bases. Then, the conjugate was detached from the solid support using NH₄OH and purified by preparative reverse phase HPLC using a triethylammonium acetate buffer. The dried sample was treated with 1,4-dioxane solution containing 3% CF₃CO₂H and 1% *m*-cresol (v/v/v %). After 30 min, the deprotected conjugate was precipitated and washed with excess diethyl ether. The deprotected conjugate was finally purified by preparative anion exchange HPLC using a Tris-HCl buffer. As shown in Figure 4, neomycin-conjugated DNA elutes faster than the unmodified DNA. The identity of the conjugate (N-dY) was confirmed by MALDI-TOF mass spectrometry (MS (MALDI-TOF) *m/z* calcd [M + H]⁺ 2795, found -2796.21, Figure 5).

Evidence of Incorporation of a Modified Base. The base composition of the conjugate was determined by complete enzymatic hydrolysis using snake-venom phosphodiesterase followed by alkaline phosphatase and subsequent reverse phase HPLC chromatography. The modified and unmodified oligonucleotides (0.2 A₂₆₀ unit) were subjected to digestion with snake venom phosphodiesterase (10 units/mL) and alkaline phosphatase (100 units/mL) in 50 μ L of 50 mM Tris-HCl buffer



Figure 3. A model of neomycin–7mer DNA conjugate. The linker is shown in yellow and neomycin is shown in atom colors. The molecule was generated from a NMR solution structure file (PDB: 124D). The structure was optimized with *Maestro* program using the AMBER* force field to a gradient of 0.05 kJ/mol Å. The continuum GB/SA model of water, as implemented in *MacroModel*, has been used in all calculations. The force field atomic charges were used for the hybrid duplex. Neomycin was built and optimized in *MacroModel* as described previously (21).

(pH 7.2) containing 10 mM MgCl_2 at 37 °C for 24 h. The reaction mixtures were analyzed by reverse phase HPLC. As shown in Figure 6, an extra peak appears at approximately 10 min, which integrates to a base ratio of 1 (X = dU–neomycin conjugate) compared to base ratio of 4 (dC) to 2 (dT). In the unmodified DNA, only two peaks elute, with ratios of 4(dC)/3(dT).

Spectroscopic Studies of Interaction of Single Strand RNA with N–Neo Conjugate. We then investigated using UV thermal denaturation studies to determine if the stabilization shown by neomycin can also be observed with the neomycin–DNA conjugate. Covalent conjugation of a large molecule like neomycin can easily lead to unfavorable base/backbone contacts, if the molecule is not placed in the major groove with needed flexibility. The thermal denaturation of the rR•dY1 duplex

(Scheme 3) in the absence and presence of 4 μM of neomycin was carried out in the presence of various concentrations of sodium ions (Table 1). In the absence of NaCl, the hybrid duplex (rR•dY1) melts at 26.3 °C. As the NaCl concentration was increased (0–100 mM), the T_m of the hybrid duplex increased from 26.3 °C to 30.0 °C. With 60 mM NaCl, the hybrid duplex showed a T_m of 28.1 °C. The presence of 4 μM of neomycin at 60 mM NaCl increases the T_m of the hybrid duplex (rR•dY) by 3.2 °C (Figure 7). When the DNA strand (dY1) was replaced with the modified oligonucleotide–neomycin conjugate (N–dY), the ΔT_m of the hybrid duplex (rR•N–dY) was 6.9 °C (with 60 mM NaCl) compared to the hybrid duplex (rR•dY1) without neomycin. Control experiments with just the amino linker led to a 1.8 °C change in T_m (see Supporting Information).

Sequence Specificity of the Conjugate. The effect of neomycin on the presence of a mismatch in this duplex (mismatch present on strand-rR¹) was also carried out. The mismatch was chosen on the RNA base which is complementary to the modified base. The presence of a single mismatch on the RNA strand decreases the melting temperature of the hybrid duplex (rR¹•dY1) from 35.3 °C to 8 °C when there is 4 μM of neomycin and 60 mM NaCl present in the solution. The mismatch penalty was similarly observed with the hybrid duplex (rR¹•N–dY) (with neomycin–oligonucleotide conjugate (N–dY) as one of the DNA strands) showing a T_m of 8 °C. This clearly suggests that neomycin's presence does not disrupt the Watson–Crick hybridization and the stabilization seen by neomycin is dependent on the retention of this fidelity leading to the A-form hybrid duplex.

Conformation of the Resulting Hybrid Duplex: Circular Dichroism (CD) Studies. The interaction of the aminoglycoside with hybrid nucleic acids can be monitored by CD spectroscopy. Depending upon the nature of the spectrum obtained from the CD scan, one can predict whether the complex exists in A-, B-, or canonical forms. The literature shows the preference of aminoglycosides for nucleic acids with A-like conformation (55–58). In some cases, transition of B- to A-conformation has also been observed with aminoglycosides, a phenomenon observed with cationic ligands such as spermine and $\text{Co}(\text{NH}_3)_6^{3+}$ (59, 60). In a recent report, a complex formed between an 8-base-pair hybrid duplex and paramomycin was shown to exist in an A-like canonical conformation (61). Our earlier competition dialysis results have shown the preference of neomycin conjugates for A-form nucleic acids. The CD spectra of both the hybrid duplexes ((rR•dY1 and (rR•N–dY) were therefore analyzed. The spectra show that the conjugate shifts the maximum in the CD spectrum from 275 nm to 271 nm when compared to nonconjugated hybrid duplex (Figure 8). This observation suggests that the conjugate hybrid duplex prefers to be in canonical A-form (61–64) and retains the form exhibited by the unmodified RNA•DNA hybrid duplex.

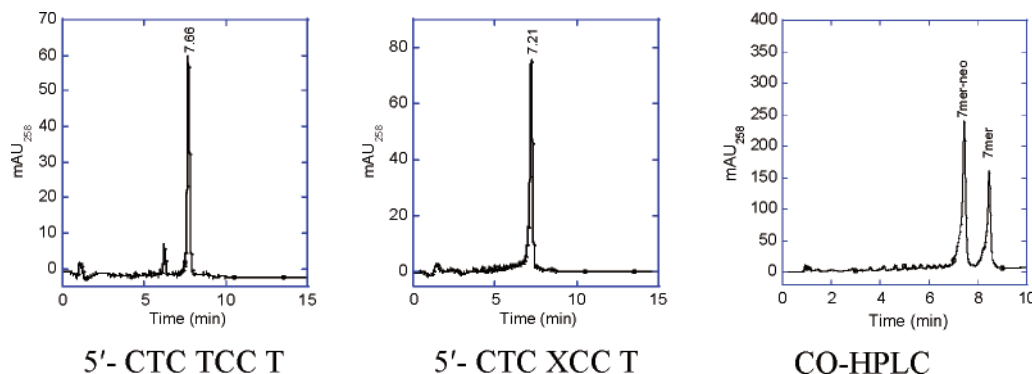


Figure 4. Conditions. Buffer A: 25 mM tris-HCl and 1 mM EDTA, pH = 8.0. Buffer B: buffer A + 1 M NaClO_4 , pH = 8.0. 2% buffer B in buffer A for 2 min; 2–40% during 15 min; flow rate was 0.75 mL/min. X corresponds to deoxyuridine–neomycin conjugate base.

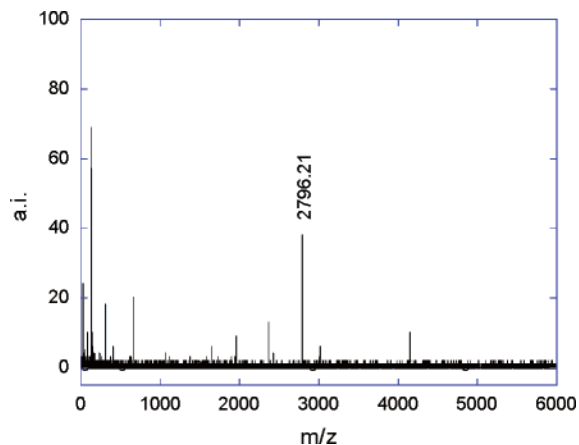


Figure 5. MALDI-TOF profile of 7mer–neomycin conjugate recorded with picolinic acid as the matrix and ammonium tartarate as the co-matrix.

Thermodynamic Basis of Interaction of Neomycin with the Target: Isothermal Titration Calorimetry.

The effect of neomycin binding to the host rR•dY duplex was also studied using ITC experiments. The experiment was conducted at 15 °C in sodium cacodylate buffer at pH 6.0. Each injection gave the corresponding heat burst curve, and integration of the areas under these curves corresponds to the associated injection heats. These injection heats were subtracted from the dilution heats, which were obtained by two separate blank titrations of the corresponding drug vs buffer and the duplex vs buffer.

The thermodynamic properties for the binding of the conjugate N-dY with RNA (rR) were determined with the help of isothermal calorimetry. Monophasic transition was observed when N-dY conjugate was titrated against rR, and the injection heat data was fit with a model for a single binding site (Figure

Scheme 3. Base Sequences Used in the Present Study^a



^a X indicates the presence of neomycin conjugated to 2'-deoxyuridine at the 5-position.

Table 1. UV Melting Profiles of the 7-mer Hybrid Duplex in the Presence of Various Concentrations of NaCl^a

	concn of NaCl (mM)	<i>T_m</i> (°C)		
		without neomycin	with 4 μM neomycin	with N-Neo-C
1	0	26.3	30.0	32.1
2	10	27.1	31.1	32.1
3	25	27.1	31.1	32.3
4	60	28.1	31.3	35.0
5	100	30.0	31.0	36.1

^a Solution conditions: [DNA] = 4 μM per strand; 10 mM sodium cacodylate buffer, 0.5 mM EDTA, pH 7.0. Samples were heated from 5 to 90 °C at 0.2 °C/min, and were brought back to 20 °C at a rate of 5 °C/min.

9). For comparison, a titration of rR against nonconjugated DNA (dY1) was also carried out, and the results are presented in Table 2.

The results show that rR single strand prefers to form the duplex approximately two times more with the N-dY conjugate than the nonconjugated dY single strand. A 2-fold change

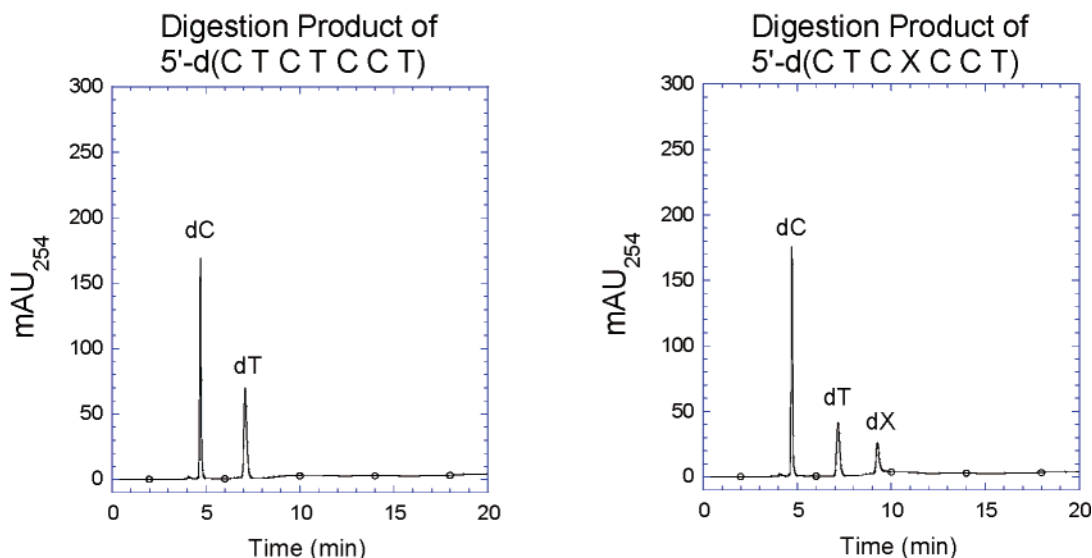
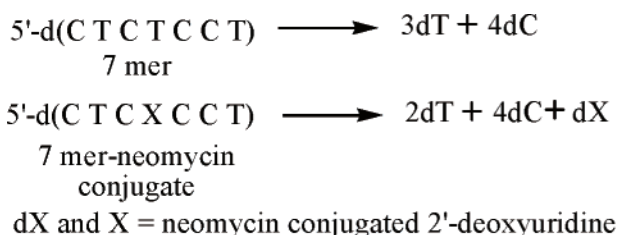


Figure 6. Conditions. Buffer A: 100 mM triethylammonium acetate and 5% of acetonitrile. Buffer B: 95% of acetonitrile and 5% of 100 mM triethylammonium acetate, pH = 7.0; 1–10% of buffer B over buffer A during 15 min, flow rate was 0.5 mL/min; 10–23% during 8 min, flow rate was 1.0 mL/min.

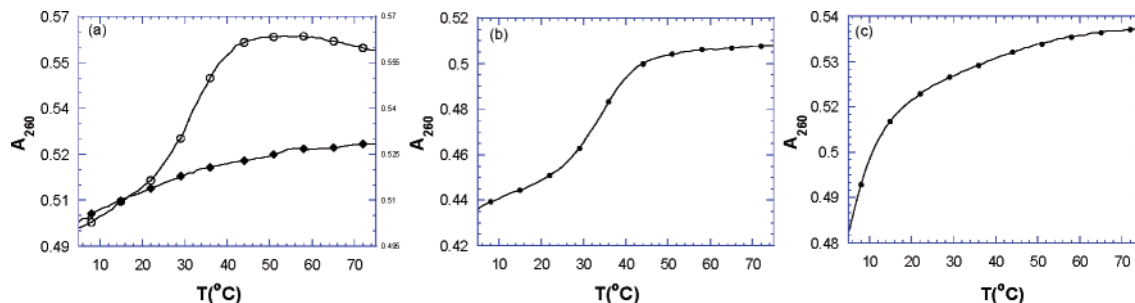


Figure 7. UV melting profiles of (a) rR•dY1 hybrid duplex in the absence (filled squares) and presence of 4 μM of neomycin (open circles); (b) rR•N-dY hybrid duplex; (c) rR¹•N-dY hybrid duplex. The spectra were recorded at 260 nm in the presence of 60 mM Na⁺ ion concentration. [DNA] = 4 μM /strand. Buffer conditions: 10 mM sodium cacodylate, 0.1 mM EDTA, pH 7.0. The melting rate was 0.2 $^{\circ}\text{C}/\text{min}$.

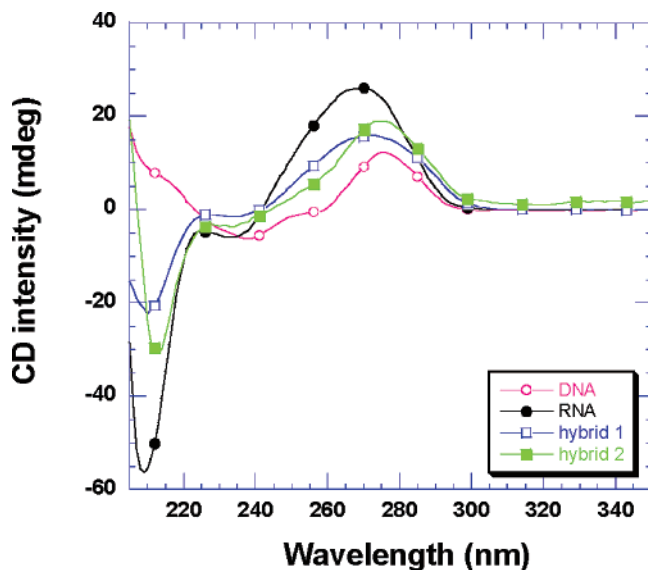


Figure 8. CD scans of 10 μM DNA duplex (dR•dY1, open circle), RNA duplex (rR•rY, filled circle), hybrid 1 (rR•N-dY, open square), and hybrid 2 (rR•dY1, filled square) at 10 $^{\circ}\text{C}$. Buffer conditions: 10 mM sodium cacodylate, 0.5 mM EDTA, 60 mM total Na⁺, and pH 7.0.

reflects the synergistic effect of DNA and covalently bound neomycin on the duplex structure. The increased stability of the duplex is driven mainly by a larger and more negative enthalpy $\{\Delta\Delta H_{\text{obs}} = -7.21 \text{ kcal/mol} (\Delta H_{\text{neomycin conjugated}} - \Delta H_{\text{nonconjugated}})\}$. This increase in enthalpy matches the enthalpy of interaction of aminoglycoside binding to the hybrid duplex (61). Interestingly, lowering the pH (to pH 6.0) leads to a decrease in K_a , such that the effect of neomycin conjugation is almost negligible (please see Supporting Information). Normally, when neomycin binds to a nucleic acid target, loss of pH leads to a lowering of binding-induced protonation (less favorable enthalpy of binding), but that is compensated by a favorable entropy such that the overall affinity of the drug to nucleic acid decreases slightly as the pH is lowered (please see Supporting Information) (65). Conjugation of neomycin to DNA in N-Neo-Cs leads to a similar decrease in $\Delta\Delta H$ (-7.2 kcal/mol at pH 7.0 to -5.5 kcal/mol at pH 6.0), but covalent attachment of neomycin does not allow for a favorable change in entropy as the pH is lowered. The unfavorable entropy changes in binding are dominated by the binding of larger DNA oligomer to its RNA target and are affected almost equally for dY1 and N-dY binding when the pH is lowered. The binding results of N-dY to RNA is also of interest in light of the general problem of the attribution and additivity of the binding free energies of ligand substituents. In general, if a ligand molecule A and ligand molecule B bind to a receptor, ligand A-B is then expected to bind so that the binding constant of A-B equals the product of two individual

binding constants. DNA (dY) binds to RNA with 100 nM affinity, whereas neomycin shows very little binding to small single strand RNA. Therefore, the conjugation of the two, as observed here, is not expected to result in a major change in DNA to RNA binding constant at room temperature. The increase in T_m of the duplex, as seen with the effect of neomycin on the hybrid duplex, is retained with this conjugation. Neomycin binding to the target is therefore dependent on the ability of the single strand DNA to bind single strand RNA and form the hybrid duplex. A corollary to the discussion is also that neomycin conjugation to DNA is not likely to result in a lack of sequence specificity in RNA binding. We have previously shown the ability of neomycin to stabilize DNA and RNA triplexes as well (27). We therefore investigated if conjugation of neomycin to DNA using this linker will allow for triplex stabilization (dY1•rR•dY2). Addition of a third strand DNA or RNA, in the absence of neomycin, does not result in the formation of the triplex, since such triplexes only form with much longer sequences in high salt conditions. We have previously shown the ability of neomycin to induce intermolecular DNA triplexes with at least 16 base pairs (26). Addition of high concentrations of neomycin did not induce the formation of the 7-base-pair triplex (dY1•rR•dY2 or dY1•dY•dY2). When the studies were conducted with neomycin-linked DNA, no change in the T_m (UV or CD melting; please see Supporting Information) was observed. These results substantiate our model where a shorter linker, such as used here, allows a better fit of neomycin to the hybrid duplex, whereas longer linkers are better suited for reaching into the triple Watson-Hoogsteen groove (26). The short size of the triplex also contributes to its instability, and more direct comparisons of duplex vs triplex stabilization can only be carried out with longer sequences. Nevertheless, using the appropriate linker and sequence design, one can, in principle, make the N-Neo-Cs specific for targeting a single strand RNA or a duplex DNA/RNA target. Additionally, variation of linker lengths in future studies are also likely to lead to much larger affinity increases per neomycin conjugation.

CONCLUSION

To investigate the advantage of nucleic acid based specificity coupled with aminoglycoside charge/shape complementarity, we have initiated the development of aminoglycoside-nucleic acid conjugates (N-Ag-Cs) (42, 43, 66, 67). This report focuses on the development of such conjugates and their binding to a biologically relevant target—ribosomal RNA.

In conclusion, we have synthesized a modified oligonucleotide, where an aminoglycoside is covalently linked to one of the bases of a 7-base-long oligonucleotide. When we began this work, there was considerable doubt that a large molecule such as neomycin can be placed in the major groove of an RNA duplex using a covalent linkage. This report is the first of its kind that shows the ability of such a large covalently linked

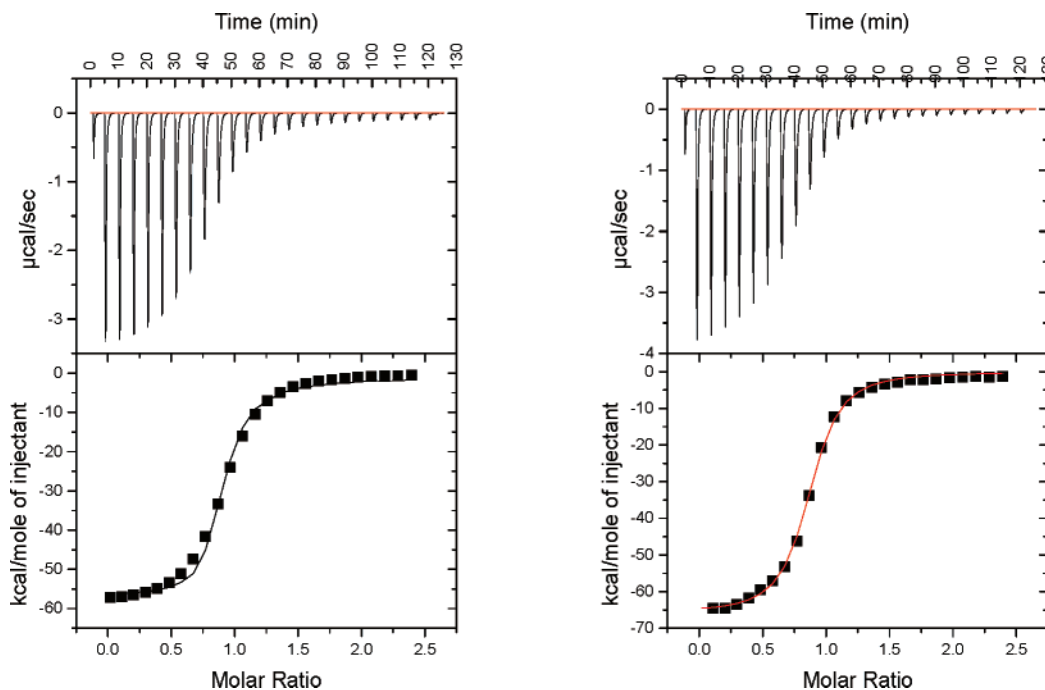


Figure 9. ITC profile of rR single strand titrated against (a) dY single strand (b) neomycin conjugate (N-dY) at 10 °C and pH 7.0 in sodium cacodylate buffer. Buffer conditions: 10 mM sodium cacodylate, 0.1 mM EDTA, pH 7.0 and 60 mM total Na^+ ions. Each heat burst curve is a result of a 10 μL injection of 200 μM of rR into 15 μM of N-dY solution. A plot of corrected ITC injection heats as a function of the [rR]/[N-dY] ratio (r_{dup}). The heats were derived by subtraction of the heats obtained by the titration of rR SS against the N-dY SS with the heats obtained by blank titration of the corresponding buffer vs buffer. The data points reflect the experimental injection heats, while the line denotes calculated fit using a model for one set of binding.

Table 2. Binding Parameters for the Complexation of the rR SS (single strand) with N-dY SS in Cacodylate Buffer at pH 7.0 and a Temperature of 10 °C Obtained from ITC

duplex	N	K_a (M^{-1})	ΔH_{obs} (kcal/mol)	$T\Delta S$ (kcal/mol)
rR•dY	0.97 ± 0.01	$2.8 \times 10^6 \pm 3.8 \times 10^5$	-58.7 ± 0.9	-50.4
rR•N-dY	0.93 ± 0.01	$5.0 \times 10^6 \pm 2.3 \times 10^5$	-65.9 ± 0.5	-54.5

carbohydrate (neomycin) to aid in RNA•DNA hybrid complexation. In addition, we have demonstrated that the N-Neo-conjugate and a 7-base-long RNA sequence present in the α -sarcin loop can form a stable hybrid duplex. The stability of the rR•dY1 hybrid duplex with 1 equiv of neomycin is comparable to the stability of the rR•N-dY hybrid duplex. The presence of a single mismatch decreases the melting temperature of both neomycin conjugated and nonconjugated hybrid duplexes. This is an important finding, as it supports the fact that association of the conjugate is driven by DNA-RNA base-pair association and not through non-sequence-specific electrostatic interactions between neomycin and the phosphodiester linkages. The linker length used here does not induce the formation of the short triplexes and will thus be specific for RNA targeting as opposed to duplex DNA targeting. Since the formation of such hybrid duplexes can be used to control gene expression by inhibition of the steps of translation, slicing, or by activation of RNase H leading to degradation of the mRNA, these conjugates have potential applications that include drug and oligonucleotide delivery to a specific RNA site.

The design, construction, and preliminary analysis of a new class of carbohydrate-DNA conjugates, which we term nucleo-aminoglycoconjugates (N-Ag-C), has been described. The example shown here—a nucleoneomycinconjugate (N-Neo-C)—illustrates that these aminoglycoside-DNA hybrids are efficiently synthesized, chemically stable, and behave similarly to an unmodified DNA strand in solution. The synthetic methodology described here allows for inclusion of any number of aminoglycoside (neomycin) molecules per oligonucleotide. These conjugates can provide a platform for the development

of a new form of hybrid biomaterials. They combine the unique specificity of nucleic acids and the recognition/binding ability of the aminoglycosides. N-Neo-Cs could have enormous potential in oligonucleotide therapeutic strategies (duplex as well as triplex approaches) and in the engineering of novel DNA-based materials. These applications will be further investigated and reported in due course.

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Supporting Information Available: UV and CD scans/melts, synthesis and characterization of intermediates (23 pages). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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