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Molecular recognition of a DNA:RNA hybrid: Sub-nanomolar binding by a neomycin–methidium conjugate

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ABSTRACT

A novel neomycin–methidium conjugate was synthesized. The covalent linkage of the aminoglycoside to an intercalator, a derivative of ethidium bromide, results in a new conjugate capable of selective recognition of the DNA:RNA hybrid duplex. Spectroscopic methods: UV, CD, fluorescence, and calorimetric techniques: DSC and ITC were used to characterize the sub-nanomolar binding displayed by the conjugate for the DNA:RNA hybrid duplex, poly(dA):poly(rU).

DNA:RNA hybrid structures have attracted recent attention due to the key roles they play in a number of biological processes. DNA:RNA hybrids can be found in transcription,[1] reverse transcription[2,3] and in the priming of DNA prior to replication.[4,5] DNA:RNA hybrids play crucial roles in different types of enzymatic activity, notably telomerases[6] and HIV RNase. However, when one attempts to identify small molecule binders of DNA:RNA hybrids, fewer than 10 pharmacophores can be found.[7–9] In fact, few reported attempts have been made to target the unique structure of DNA:RNA hybrids and only a handful of molecules have been identified.[8] For comparison, the number of molecules studied that target duplex DNA is in the thousands. A few years ago, we identified aminoglycosides as lead candidates in the groove-based recognition of A-form nucleic acid structures.[2] DNA:RNA hybrids normally adopt A-form structures. These major groove binders were subsequently shown to bind DNA:RNA hybrids in the sub-nanomolar range and inhibit RNA processing enzymes, RNase H and RNase A, by binding to RNA:DNA hybrids.[10] Two other laboratories, using two different assays, have shown the preference of ethidium bromide for DNA:RNA hybrids,[9,11] which binds in the low μM range.

We hypothesized that by conjugating neomycin with a specific DNA:RNA binding intercalator, ethidium bromide, high selectivity and binding of DNA:RNA hybrids can be achieved. Furthermore, a new class of molecules with high DNA:RNA hybrid affinity can be developed. A neomycin–methidium conjugate (NM, Scheme 1, compound **3**) has therefore been synthesized by formation of an amide bond linkage between neomycin and methidium carboxylic acid, **2**. We report that **3** is more potent in stabilizing DNA:RNA hybrids than neomycin, ethidium bromide, or a non-covalent combination of both. In fact, the affinity of the conjugate parallels the highest affinity aminoglycoside ligands ever identified for nucleic acid recognition.

The following assumptions were made in the design of the conjugate: the amino groups on rings I, II, and IV of neomycin are necessary in stabilizing and recognizing the nucleic acid grooves (aminoglycosides without any of these amines do not stabilize nucleic acids as efficiently).[7,12–14] The 5″ –OH on ring III was thus chosen to provide linkage to the intercalating unit. The intercalator 6-(4-carboxyphenyl)-3,8-diamino-5-methylphenanthridinium chloride (**2**) was linked to neomycin amine, **1**, (prepared in three steps from neomycin)[15–17] using DCC-mediated amide bond formation. TFA deprotection yielded the conjugate, **3**, in good yields.

CD and UV melting experiments were carried out to confirm hybrid duplex stabilization in the presence of the ligands. Addition of 2.2 μM of neomycin {ratio of poly(dA):poly(rU) base pairs to drug (r_{bd}) = 9} and ethidium bromide leads to a T_m increase of 12 °C, whereas addition of **3** at the same r_{bd} leads to a T_m increase of 20 °C (Fig. 1a). Increased concentration of **3** to the DNA:RNA hybrid resulted in a gradual increase in the melting temperature of the

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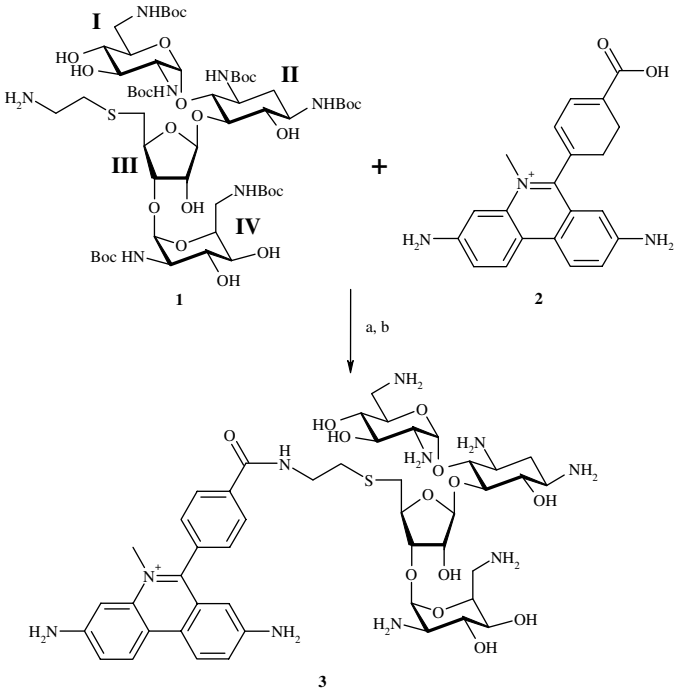

Scheme 1. Reagents and conditions: (a) DCC, DMAP, DMF, rt, 28 h, 80%; (b) TFA/CH_2Cl_2, $HSCH_2CH_2SH$, rt, 30 min, 90%.

hybrid. As depicted in Figure 1b, as the concentration of **3** is increased from 0 to 3 μM the melting temperature of poly(dA):poly(rU) increases from 48.9 to 70.1 °C, a 21.2 °C increase overall.

In order to ascertain the selectivity of this novel conjugate, nucleic acid structures known to bind ethidium bromide and neomycin were thermally denatured in the presence of equimolar amounts of **3**, affording a thermal melting profile for the conjugate, Table 1. Additionally, a mixture composed of various equimolar nucleic acids was thermally denatured in the presence of **3** to further illustrate selectivity of the conjugate, Figure 2. A relationship between binding preference and thermal stabilization afforded by **3** was obtained using UV melting experiments. Table 1 shows those nucleic acids which **3** displays a high preference for and shows significant thermal stabilization. Poly(dA):poly(rU) stability is increased by 46.9 °C, 12.6 °C higher than any other nucleic acid

Table 1
Melting temperatures of nucleic acid structures (20 μM/bp) with NM, **3**, (2.2 μM)

	T_{m0} (°C)	T_m (°C)	ΔT_m (°C)
A-site RNA	71.7	79.8	8.1
Calf thymus DNA	86.9	>95	>8.1
Poly(dA):2poly(dT)	23.1	–	–
	68.2	75.9	7.7
Poly(dA):poly(dT)	68.4	76.1	7.7
Poly(dA–dT):poly(dA–dT)	67.9	75.3	7.4
Poly(dG):poly(dC)	93.6	>95	–
Poly(rA):poly(rU)	57.1	91.4	34.3
Poly(dA):poly(rU)	43.6	90.5	46.9
Poly(rA):poly(dT)	62.3	82.4	20.1

Buffer: 10 mM sodium cacodylate, 0.5 mM EDTA, pH 5.5 and 100 mM NaCl.

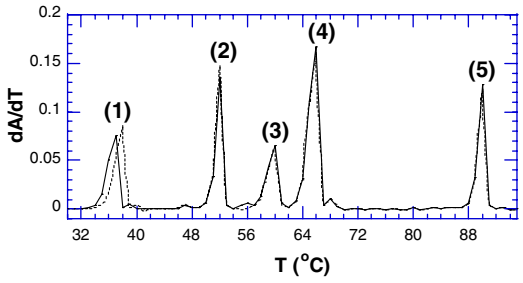

Figure 2. Mixed melting profile of various nucleic acids. The panel shows a derivative plot for the mixed melting of poly(dG):poly(dC); peak 5, poly(dA):poly(dT); peak 4, poly(rA):poly(dT); peak 3, poly(rA):poly(rU); peak 2, poly(dA):poly(rU); peak 1. Individual polynucleotide concentration was 10 μM/bp, NM, **3**, (r_{bd} = 20). In the panel, the solid line reflects native melting and the dashed line represents the melting with NM. Buffer: 1.5 mM Na_2HPO_4, 0.5 mM NaH_2PO_4, 0.25 mM Na_2EDTA and 46.25 mM NaCl at pH 7.0.

structure. Not only does **3** show a high preference for DNA:RNA hybrids, poly(dA):poly(rU) thermal stabilization afforded by **3** is significantly higher than any other nucleic acid structure.

Most recently, an assay using thermal denaturation of mixtures containing various nucleic acids in the presence of ligand was developed to determine structural and sequence selectivity.[18] As seen in Figure 2, when assayed against a solution containing poly(dA):poly(rU), poly(rA):poly(dT), poly(dA):poly(dT), poly(rA):poly(rU), and poly(dG):poly(dC), at r_{bd} = 20, only the melting temperature of poly(dA):poly(rU) is increased, while all other duplexes remain unchanged. In fact, increasing the concentration to r_{bd} = 10 affords stabilization to poly(dA):poly(rU) (2.0 °C) and

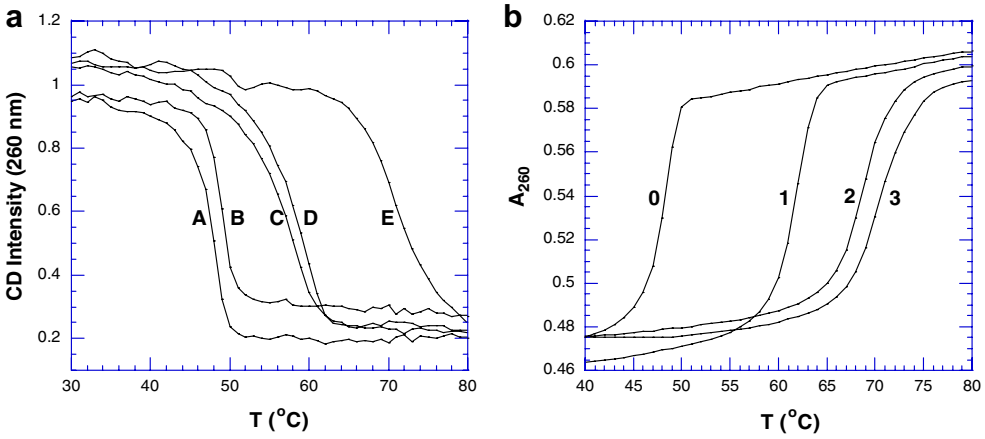

Figure 1. (a) CD melting profile of poly(dA):poly(rU) in the presence of ligands. DNA:RNA hybrid (A) (20.0 μM/bp) was mixed with ethidium bromide (B), neomycin (C), neomycin, and ethidium bromide (D) and NM, **3**, (E). All ligands were at a concentration of 2.2 μM. (b) UV melting profile of poly(dA):poly(rU) in the presence of increasing **3**. DNA:RNA hybrid (20.0 μM/bp) was mixed with NM **3** at increasing concentrations (0, 1, 2, and 3 μM) before slow heating to 90 °C at a rate of 10 °/h. Buffer conditions: 10 mM sodium cacodylate, 0.5 mM EDTA, 100 mM NaCl, and pH 6.8.

poly(rA):poly(dT) (0.9 °C), see Supporting Information. Convenient in this strategy, is the combination of both ligand selectivity and thermal stabilization afforded by **3**. The smaller changes in T_m, as observed in the mixed melting experiments, are consistent with the previously reported data.[18] Provided, is irrefutable evidence of NM preference for poly(dA):poly(rU). The difference in stabilization of the two hybrids by **3** can be attributed to the conformational differences adopted by the two hybrids of which poly(dA):poly(rU) adopts a more A-like conformation than poly(rA):poly(dT).[19–21]

Theoretically, if one looks at the product of the K_a values for methidium and neomycin, one expects a K_a in the $10^{12}\ M^{-1}$ range (Table 2). There are very few methods which can accurately determine such large binding constants. Fortunately, a calorimetric method has previously been used to determine K_a in this range.[22]

The apparent ligand–DNA:RNA hybrid association constants were estimated using the following equations. The melting temperature difference in the absence and presence of drug was used to estimate the association constant, K_{T_m}, using the following equation[23]

$$\frac{1}{T_{m0}} - \frac{1}{T_m} = \frac{R}{n\Delta H_{wc}} \ln(1 + K_{T_m} L). \quad (1)$$

In Eq. (1), terms T_{m0} and T_m represent the melting temperatures of the native hybrid without and with ligand, as determined with thermal denaturation, UV and CD. The number of drug molecules bound per duplex, n, was determined using titrations of drug into duplex (fluorescence for ethidium bromide and **3** and CD for neomycin). ΔH_{wc}, Watson–Crick duplex melting enthalpy, was determined using DSC. L is the free drug concentration at T_m, estimated at one-half the total drug concentration. The binding constant at the melting temperature, T_m, was extrapolated to a reference temperature (T) of 20 °C using the integrated van't Hoff equation[24]

$$K_T = \frac{K_{T_m}}{e^{-\Delta H_T/R(1/T_m - 1/T)} e^{\Delta C_p T/R(1/T_m - 1/T)\left(\frac{T_m}{T}\right)^{\Delta C_p/R}}}, \quad (2)$$

where ΔH_T was determined experimentally using ITC, R is the gas constant, and ΔC_p was determined using the following equation[25]

$$\Delta C_p = \frac{\Delta H}{\Delta T}. \quad (3)$$

Enthalpy values (ΔH) were determined using binding enthalpies from excess site ITC titrations (used to identify the heat of interaction of the primary high affinity site), at various temperatures. Enthalpy values were obtained by subtraction of the heat of dilution of ligand into buffer, see Supporting Information, from the sample titration of ligand into duplex. We have calculated K_T for neomycin, ethidium bromide, and **3** using this approach. The data shown in Table 2 illustrate the power of conjugating two ligands that bind to a receptor at different sites. Neomycin binds to the hybrid duplex with a $10^6\ M^{-1}$ affinity, while ethidium bromide binds the hybrid duplex with a similar affinity at $10^6\ M^{-1}$. The conjugate shows a 10,000-fold improvement over neomycin, with an affinity of $4.8 \times 10^{10}\ M^{-1}$.

In an effort to compare the affinity of **3** to other nucleic acid structures, K_T values were calculated for both the RNA and DNA duplexes, Table 2. The conjugate binds the DNA duplex poly(dA–dT)$_2$ with an affinity of $1.0 \times 10^6\ M^{-1}$ and the RNA duplex poly(rA):poly(rU) with an affinity of $2.1 \times 10^9\ M^{-1}$. The affinity for the RNA duplex, to **3**, is higher than its affinity for both neomycin ($10^7\ M^{-1}$) and ethidium bromide ($10^6\ M^{-1}$). The conjugate, when binding the duplex DNA, displays a moderate increase in affinity, when compared to the DNA duplex affinity for neomycin ($10^4\ M^{-1}$), and ethidium bromide ($10^5\ M^{-1}$). More significant is the conjugates selectivity for the hybrid duplex over the RNA duplex and DNA duplex. Compound **3** binds to the hybrid poly(dA):poly(rU) with 20-fold higher affinity than the RNA:RNA duplex, and almost 40,000 higher affinity than the DNA:DNA duplex. Compound **3** clearly displays the power of conjugation and expansion of aminoglycoside based binding to non-traditional targets. In fact, not only does the conjugate bind non-traditional target, poly(dA):poly(rU), the affinity for this duplex is much higher than the reported neomycin binding to the 16S A-site RNA ($9.1 \times 10^8\ M^{-1}$).[26]

The experiments were conducted at pH 5.5 to eliminate binding induced drug protonation effects and to determine intrinsic enthalpies of interaction between drug and polymer. Protonation effects on aminoglycoside binding have been previously studied[22] and binding-linked protonation leads to an increase in observed binding enthalpy. In fact, it has been shown that a decrease in pH leads to a moderate increase in affinities of aminoglycosides to their nucleic acid targets (3- to 5-fold increase from pH 7.0 to pH 6.0 in aminoglycoside binding to the DNA:RNA hybrid and rRNA A-site).[10,22] Therefore, the affinities described here are only slightly higher than the affinities that would be observed under physiological conditions. Furthermore, low salt concentration, 100 mM NaCl, was used to isolate the primary, high affinity, binding site.

Table 2
Thermodynamic profile of nucleic acid interactions with NM, **3**, neomycin and ethidium bromide

	Poly(dA):poly(rU)			Poly(rA):poly(rU)			Poly(dA–dT):poly(dA–dT)		
	NM (**3**)	Neomycin	EtBr	NM (**3**)	Neomycin	EtBr	NM (**3**)	Neomycin	EtBr
ΔH_{wc}[a]	3.84	3.84	3.84	6.34	6.34	6.34	3.90	3.90	3.90
T_{m0}[b]	43.0	43.0	43.0	53.4	53.4	53.4	62.4	62.4	62.4
T_m[b]	90.5	65.8	61.8	77.0	64.7	64.2	66.2	62.9	63.2
n[c]	9.7	6.5	4.6	9.6	8.0	2.5	9.1	7.3	5.3
$\Delta H_{(15\,°C)}$[d]	4.4 ± 0.1	3.6 ± 0.1	−5.6 ± 0.1	3.3 ± 0.1	1.8 ± 0.1	−5.8 ± 0.1	1.2 ± 0.1	−1.1 ± 0.1	−3.7 ± 0.1
$\Delta H_{(20\,°C)}$[d]	1.9 ± 0.1	2.7 ± 0.1	−5.9 ± 0.1	1.9 ± 0.1	0.7 ± 0.1	−6.2 ± 0.1	0.8 ± 0.1	−1.1 ± 0.1	−4.5 ± 0.1
$\Delta H_{(25\,°C)}$[d]	1.1 ± 0.1	2.1 ± 0.1	−6.5 ± 0.1	0.8 ± 0.1	−0.5 ± 0.1	−6.5 ± 0.1	0.2 ± 0.1	−1.3 ± 0.1[g]	−5.1 ± 0.1
ΔC_p[e]	−339 ± 16	−150 ± 13	−93 ± 7	−256 ± 9	−223 ± 11	−90 ± 9	−104 ± 12	−29 ± 16	−139 ± 6
$K_{T(20\,°C)}$[f]	$(4.8 \pm 0.1) \times 10^{10}$	$(9.9 \pm 0.1) \times 10^6$	$(9.3 \pm 0.1) \times 10^6$	$(2.1 \pm 0.2) \times 10^9$	$(2.6 \pm 0.1) \times 10^7$	$(1.8 \pm 0.1) \times 10^6$	$(1.0 \pm 0.1) \times 10^6$	$(7.0 \pm 0.5) \times 10^4$	$(2.0 \pm 0.1) \times 10^5$

[a] Data obtained from DSC melting profiles (kcal/mol).
[b] Data obtained from CD and UV melting profiles (°C).
[c] Data obtained from titrations, as outlined in text.
[d] ΔH is corrected binding heat (kcal/mol) derived by integration of heat burst curve from the sample titration, followed by subtraction of the dilution head from the control titration.
[e] ΔC_p calculated from Eq. (3) (cal/mol K).
[f] Binding affinities calculated from Eqs. 1–3 (M^{-1}). ΔH values were recorded at 15, 20, and 25 °C (for the titration of neomycin into poly(dA–dT)$_2$, ΔH values were also recorded at 30 °C, see Supporting Information). Buffer: 10 mM sodium cacodylate, 0.5 mM EDTA, 100 mM NaCl, and pH 5.5.

Conjugation leads to a much more negative ΔC_p of interaction (−339 cal/mol K) than neomycin or ethidium bromide binding to the polymer. ΔC_p values can be impacted by a number of factors including the release of constrained water molecules from the hydration shell,[27] binding induced changes in internal vibrational modes,[28] electrostatic interactions[29] and the temperature dependent equilibrium, such as protonation, upon drug uptake.[30] It has previously been shown that groove binders exhibit negative ΔC_p values, a result attributed to the displacement of large amounts of non-polar surface area.[31,32] However, Barbieri, et al. suggest that aminoglycosides do not disrupt the spine of hydration,[33] even at pH 5.5, and the observed negative ΔC_p value is unrelated to change in solvent accessible areas. Perturbations to helical structure through disruption of adenine base stacking has been observed upon aminoglycoside binding to the A-site and is the main contributor to observed negative ΔC_p values.[30,33] Further work will help assign the exact contribution of these effects in the ΔC_p values observed here.

Previous studies have shown the preference of aminoglycosides to RNA:RNA duplex over the DNA:RNA hybrid.[10] Our work shows how conjugation to small molecules, with alternate modes of binding, can be used to alter the structural selectivity of aminoglycosides. Even though the conjugate does not display the energetic additivity estimated from the individual binding moieties, the sub-nanomolar binding affinity remains very significant and far exceeds the affinities of any known DNA:RNA binding drugs. Further studies using different linker modifications should allow us to optimize the affinity of the conjugate.

The conjugate reflects the first example of an aminoglycoside ligand that binds to a nucleic acid target with affinities much higher than the affinities shown for traditional aminoglycoside–nucleic acid targets. A recent report in C&E News cites a critical need for development of such high affinity DNA:RNA binders.[34] This work should allow one to translate the recognition event to therapeutically relevant DNA:RNA hybrids sequences such as telomerase and RNase H inhibitors.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.05.090.

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