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Binding studies of cationic uridyl ribonucleic guanidine (RNG) to DNA

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Abstract—Replacement of the phosphodiester linkages of polyanionic RNA with guanidine linkers provides a polycationic ribonucleic guanidine (RNG). The pentameric uridyl RNG (RNG U5) was found to bind a pentameric adenyl DNA (DNA A5) with a 1:1 stoichiometry as determined by the method of continuous variation. This polycationic RNG binds with unprecedented affinity with the polyanionic DNA providing a double helix. This association of RNG and DNA is highly sequence specific. Thermal denaturation (T_m) studies establish that RNG is able to discriminate between complementary and noncomplementary bases. Results of the hybridization properties, sequence specificity, and the global conformation studies of the RNG U5·DNA A5 duplex are described.

Putative drugs consisting of oligonucleotide (ODN) analogs capable of inhibiting cellular processes at the transcriptional or translational level via base pair interactions with DNA or RNA are known as antisense and antigene agents, respectively.[1] Key goals in the design of such agents include the following: increasing the binding affinity while maintaining the sequence specificity; resistance to degradation by cellular nucleases; and increased membrane permeability. Significant advances have been made in the study of a number of structural analogs of DNA and RNA designed to be antisense/antigene agents, by their enhancement of both stability and affinity for given sequences of DNA and RNA.[2] Replacement of the negatively charged phosphodiester backbone with neutral linkages eliminates the electrostatic repulsion that exists in duplex DNA, thus increasing binding affinity. Oligonucleotides connected by methylphosphonate,[3] methylenemethylimino (MMI),[4] and amide linkages[5] are representative of this strategy. An alternative approach involves replacing the sugar–phosphate backbone entirely, such as in the case of peptide nucleic acid (PNA),[6] phosphonic ester nucleic acid (PHONA),[7] and nucleic acid analog peptide (NAAP).[8] These oligonucleotide analogs with modified backbones are resistant to nuclease degradation.

It is well known that naturally occurring polyamines, such as spermidine and spermine, bind strongly to DNA and stabilize duplex and triplex formation. The enhanced thermal stability of the resultant complexes derives from the reduction of the anionic electrostatic repulsion between the phosphate moieties by the cationic polyamines. Incorporation of positive charges into oligonucleotides has been correctly anticipated to increase binding of analogs to DNA and RNA strands. Aminoalkyl linkers attached through the bases[9] and the sugar moieties[10] have both been synthesized and used for biological and biochemical studies.

We have reported the synthesis and binding properties of DNG, wherein the negatively charged phosphodiester linkages of DNA have been entirely replaced by positively charged guanidium linkages.[1,11] DNG binds strongly to target DNA because the repulsive electrostatic interactions of duplex DNA are replaced by close attractive electrostatic interactions in DNA·DNG duplex. From computational studies,[11a,12] DNG is anticipated to maintain its positive charge in proper alignment to maximize its interaction with the backbone of the negatively charged phosphodiester linkage of the opposite strand. A pentameric thymidyl oligomer of DNG, with four positively charged guanidium linkages, has been shown to bind to polyadenine DNA with unprecedented high affinity in a 2:1 thymine–adenine complex.[11a] Since interactions with polyguanine, cytidine, and thymine were not observed, DNG exhibits fidelity of base-pair recognition. In addition, the highly positively charged octameric

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thymidyl DNG oligomer was able to discriminate between complementary and noncomplementary base pairs. At single mismatch points, stability decreased by approximately 4 °C, while a mismatch at the center lowered it by 15 °C, as determined by thermal denaturation studies.[13] Although the double helical structure of the duplex formed between DNG and natural DNA or RNA is unknown, computational molecular modeling suggests that the DNG·DNA duplex primarily retains a B-DNA conformation while the DNG·RNA duplex adopted an A-type structure.[11b] In addition to these results, the guanidium linkage was shown to have nuclease resistance[14] and positively charged backbones may give rise to cell membrane permeability through electrostatic attraction of the guanidine moiety to the negatively charged phosphate groups of the cell surface. Because of the potential antisense/antigene aspects of guanidium linkages of DNG, further studies on the synthesis and properties of guanidium linked oligonucleotides are warranted.

The interesting differences and similarities between the nature of DNA and RNA prompted us to explore the synthesis and properties of RNG. Even though RNA possesses a strong affinity for DNA, its susceptibility to various nucleases and the labile nature of the phosphodiester backbone limit its application.[15] RNG should be better suited as an antisense/antigene agent because the guanidium linkages are neither susceptible to cellular nuclease nor to chemical degradation under physiological conditions. Recently, we described the synthesis of the pentameric uridyl RNG[16] (Fig. 1) and provided modeling studies involving oligomeric RNG complexed with DNA.[17] Molecular modeling studies of the double-stranded RNG·DNA [RNG U8·DNA A8] suggested that the guanidium backbone in RNG was a viable alternative to the phosphate linkages of RNA.[17] We report here the DNA binding properties of the pentameric uridyl RNG (RNG U5) and provide results which demonstrate that pentameric uridyl RNG forms unusually stable complexes with pentameric adenyl DNA (DNA A5), but does not form detectable structures with DNA consisting of noncomplementary bases.

The stoichiometry[18] of binding of RNG U5 and DNA A5 was determined by the method of continuous variation[19] to generate mixing curves of the absorbance versus mole fraction of RNG U5 and DNA A5 (Fig. 2). This method is based on the assumption that a decrease in absorbance is proportional to the number of base pairs hydrogen bonded between the interacting species. Increasing mole fraction of RNG U5 to the DNA A5 (pH 7.0 and $\mu = 0.10$ with KCl at 20 °C) lowered the UV absorbance at 260 nm. An inflection point at 0.5 mole fraction indicated the formation of RNG U5·DNA A5 duplex with the expected 1:1 stoichiometry.

The stability of the duplexes formed by RNG U5 and DNA A5 was studied by thermal denaturation experiments.[20] To confirm the effect of the guanidium linkage of the RNG on the thermal stability of the duplexes, the T_m values for an unmodified RNA U5·DNA A5 duplex and DNA T5·DNA A5 duplex were also determined (Fig. 3a). As expected, RNG U5 with four charged guanidium linkage binds to DNA A5 with much higher affinity ($T_m = 62$ °C) than natural RNA U5 and DNA T5 (extrapolated T_m values of 0 and −10 °C, respectively) do. Although the RNG U5·DNA A5 and RNG U5·DNA AAAAT duplexes (Figs. 3a and 4) exhibited broad melting transition states, this anomaly is similar to DNA·DNG melting curves,[13a] this can be explained by the electrostatic interaction between the positive charge of RNG and the negative charge of DNA, which provides an additional bonding, in spite of breaking down the Watson–Crick base pairing. In addition, no positive hyperchromic shift was seen in the UV spectra of a solution, between 15 and 95 °C, which contained RNG U5 and DNA T5, C5, and G5 (Fig. 3b). This is evidence against RNG binding to DNA in a nonspecific manner. Therefore, RNG maintains base-pair specificity while dramatically increasing its affinity for DNA com-

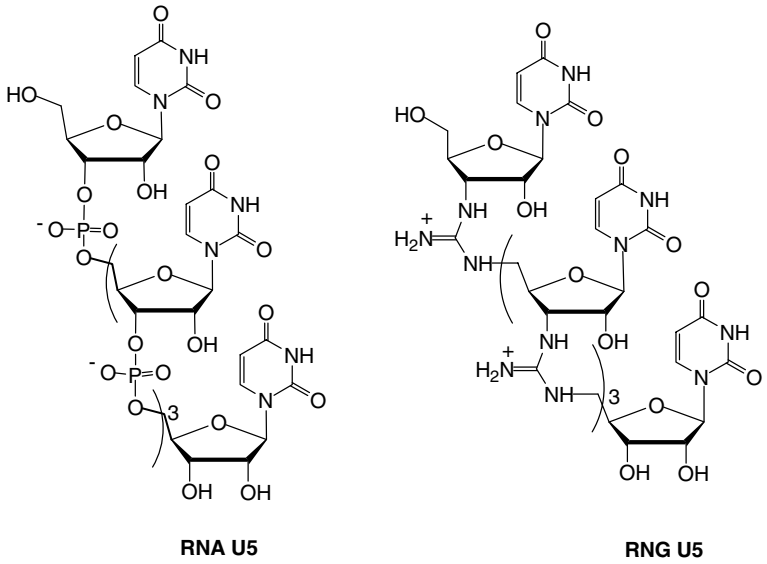

Figure 1. Structures of RNA U5 and RNG U5.

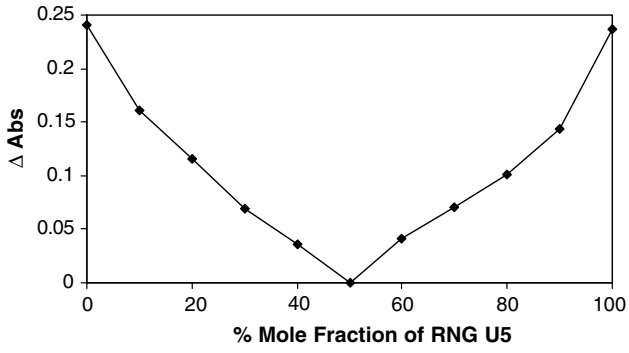

Figure 2. Job plot[19] mixing curves of the change of absorbance at 260 nm (ΔA_{260}) of aqueous solutions (pH 7.0, $\mu = 0.12$ at 20 °C) with varied mole fractions of RNG U5 and DNA A5 ([RNG U5] + [DNA A5] = 2.0 μM). The inflection point indicates the stoichiometry to be a RNG·DNA duplex.

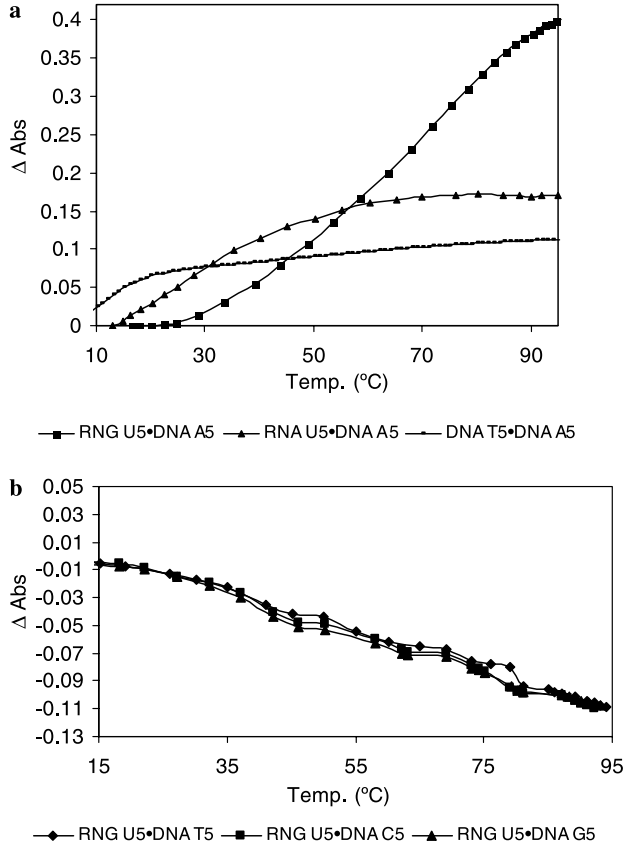

Figure 3. (a) Thermal denaturation curves for RNG U5·DNA A5, RNA U5·DNA A5, and DNA T5·DNA A5; (b) thermal denaturation curves for RNG U5 in the presence of DNA T5, DNA C5, and DNA G5. Absorbance was measured at 260 nm, [oligomer] = 4 μM, [K_2HPO_4] = 10 mM, and [KCl] = 100 mM, pH 7.0.

pared with the standard RNA·DNA complex. A negative hyperchromic shift was observed for RNG U5 in the presence of DNA T5, C5 or G5 as shown in Figure 3b. Plots of UV absorbance at 260 nm against temperature of the three DNAs with RNG are virtually the same (Fig. 3b). The results show that the nature of the nucleobases has nothing to do with this hyperchromic shift. It is probably

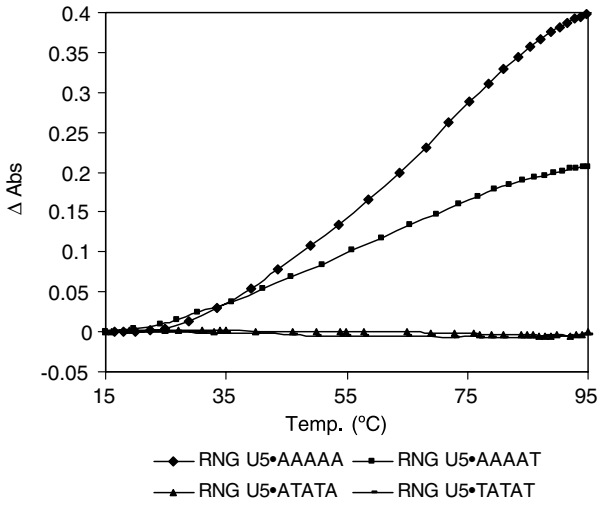

Figure 4. Thermal denaturation curves for RNG and complementary DNA and mismatched DNAs to investigate sequence specificity. Absorbance was measured at 260 nm, [oligomer] = 4 μM, [K_2HPO_4] = 10 mM, and [KCl] = 100 mM, pH 7.0.

due to an unknown conformational change of the RNG single strand resulting from interaction between ribose–phosphate backbone of DNA and guanidium backbone of RNG. In contrast, no negative hyperchromic shift was shown for RNG U5·DNA A5 and RNG U5·AAAAT duplexes (Fig. 4). This can be explained as the positive hyperchromic shift caused by RNG U5·DNA A5 and RNG U5·AAAAT duplexes melting is much higher than the negative hyperchromic shift caused by the unknown conformational change of RNG single strand.

To study the sequence specificity of the binding of RNG with complementary DNA,[20] RNG U5 was allowed to form duplexes with complementary DNA and mismatch DNAs, and the thermal stability of the resultant duplexes was monitored by thermal denaturation (Fig. 4). The RNG U5·AAAAT duplex contains one mismatched base, exhibiting a 7 °C decrease in T_m in comparison to a fully complementary RNG U5·AAAAA duplex (Table 1). In addition, DNA oligomers with two and three mismatched bases (ATATA, TATAT) did not show base-pair interaction with pentameric uridyl RNG at all. This clearly demonstrates that the binding of RNG with complementary DNA is sequence specific.

In order to describe the duplex properties more quantitatively, thermodynamic calculations were performed. The results and parameters are summarized in Table 1. Standard free energies (ΔG^0) for duplex formation are presented in Eqs. 1–3.

$$\text{DNA T5} + \text{DNA A5} \overset{\Delta G^0 = -6.9\ \text{kcal/mol}}{\rightleftharpoons} \text{DNA T5} \cdot \text{DNA A5} \quad (1)$$

$$\text{RNA U5} + \text{DNA A5} \overset{\Delta G^\circ = -7.1\ \text{kcal/mol}}{\rightleftharpoons} \text{RNA U5} \cdot \text{DNA A5} \quad (2)$$

Table 1. Melting temperatures and thermodynamic parameters for hybrid formed between RNG U5 and DNAs

	DNA T5·DNA A5	RNA U5·DNA A5	RNG U5·AAAAT	RNG U5·DNA A5
Regression equation[a]	$f = \frac{y_0 + a}{\left(1+e^{\left(\frac{-(x-x_0)}{b}\right)}\right)^c}$			
Parameters	$a = 0.41$ $b = 20.26$ $c = 40.25$ $x_0 = -95.51$ $y_0 = 0.25$	$a = 0.41$ $b = 11.24$ $c = 0.22$ $x_0 = 40.24$ $y_0 = -0.23$		
T_m (°C)	≈−10[b]	≈0[b]	55.0[c]	62.0[c]
ΔH_{VH} (kcal/mol)	−14.2	−15.7	−24.3	−27.7
ΔS^0 (cal/mol/K)	−26.2	−29.8	−48.5	−55.0
$\ln K(T)$	10.7	11.4	16.2	19.04
ΔG^0 (kcal/mol)	−6.9[d]	−7.1[d]	−9.8[e]	−11.2[e]

[a] Because DNA T5·DNA A5 and RNA U5·DNA A5 duplexes appeared to have a T_m at lower temperature limit of the experiment, T_m curves were extrapolated using regression equation.
[b] Conservative estimate of $T_m = -10$ °C, 0 °C, which were obtained from extrapolated T_m curve, was used for the calculation.
[c] The reported T_m values were an average of three experiments (±0.2).
[d] ΔG^0 values were calculated from extrapolated melting curve manually as they did not exhibit clear T_m transitions due to the low T_m values.[22] The thermodynamic data for the melting transitions of duplexes were calculated following the work of Marky and Breslauer.[21]
[e] ΔG^0 values were calculated using 'thermal software' program (van't Hoff calculation method) provided with the Cary 100-E2 9802310Z UV/VIS spectrometer automatically since they exhibit clear T_m transition state.[21] The thermodynamic data for the melting transitions of duplexes were calculated following the work of Marky and Breslauer.[21]

$$\text{RNG U5} + \text{DNA A5} \underset{}{\overset{\Delta G^{\circ} = -11.2 \text{ kcal/mol}}{\rightleftharpoons}} \text{RNG U5} \cdot \text{ DNA A5} \quad (3)$$

It reveals that there is no difference between DNA T5 and RNA U5 binding to DNA A5 ($\Delta\Delta G^0 = -0.2$ kcal/mol (Eq. 2 ΔG^0 − Eq. 1 ΔG^0)). However, the $\Delta\Delta G^0$ of −4.1 kcal/mol (Eq. 3 ΔG^0 − Eq. 2 ΔG^0) and −4.3 kcal/mol (Eq. 3 ΔG^0 − Eq. 1 ΔG^0) translates into an increase in binding over three orders of magnitude. It clearly demonstrates the tighter binding (over 10^3 times) of RNG to its DNA templates compared to RNA and DNA. This increase is attributed to the powerful attraction between the positively charged RNG guanidium groups and the negatively charged phosphates of RNA. The strength and fidelity of the uridine/adenine base pair, due to hydrogen bonds, in combination with electrostatic attraction of RNG for DNA suggests that strategically substituted guanidium-linked uridines may yield a powerful antisense oligonucleotide.

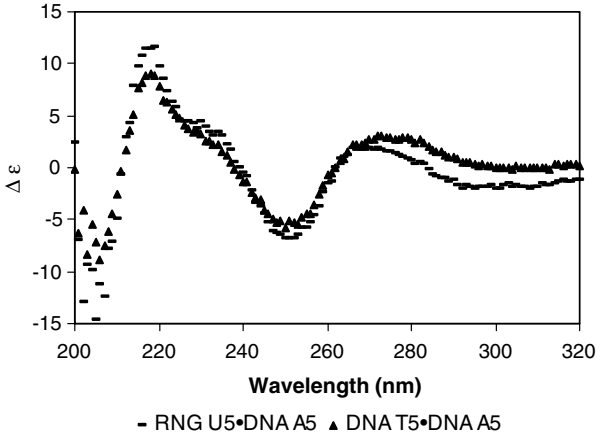

Figure 5. CD spectra of RNG U5·DNA A5 complex compared to DNA T5·DNA A5 complex. [oligomer] = 4 μM, [K_2HPO_4] = 10 mM, and [KCl] = 100 mM, pH 7.0.

The CD spectrum of an oligomer in solution can give valuable information about the global conformation of a duplex. DNA bases are planar and have no intrinsic CD signal of their own. Any CD signals observed in the absorbance band of the bases (200–300 nm) are caused by the spatial organization of the bases in a chiral structure, such as a helix, under the influence of the chiral sugar backbone. Base stacking interactions magnify this effect and give rise to the CD signals observed for DNA and RNA oligomers and polymers.[23] The spectra of the duplexes (RNG U5·DNA A5 and DNA A5·DNA T5) showed a positive CD band around 275 nm and a negative CD band around 245 nm that are attributable to B-like DNA conformation (Fig. 5).[24] The shapes of both spectra were quite similar to each other, although the intensity of the positive band in the spectrum of the duplex containing guanidium linkages RNG U5·DNA A5 was clearly less than that of the standard duplex. Molecular modeling[17] predicted that the overall structure of RNG is equilibrated at a B-DNA conformation, and the RNG strand takes on the general conformation of the DNA backbone. The loss of band intensity results from certain RNG backbone torsion angles around the guanidium group being different than the standard A-RNA values in order to relieve the tension due to the replacement of the diester phosphate linkage.

From the above discussion, the following conclusions can be drawn about RNG: (i) the pentameric uridyl RNG binds to an adenyl DNA strand in a 1:1 ratio; (ii) due to electrostatic attraction in place of electrostatic repulsion, a RNG dimeric complex with DNA is thermodynamically favored in comparison to RNA·DNA,

DNA·DNA duplexes; (iii) the pentameric uridyl RNG was able to discriminate between complementary and noncomplementary base pairs. These results suggest that RNG is a good candidate as a novel antisense molecule; (iv) The CD spectrum shows that the RNG U5·DNA A5 complex takes on a somewhat different conformation of the nucleic acid backbone than that of the RNA·DNA complex. Further studies of the thermal denaturation and global structure of RNG are currently under investigation.

Acknowledgment

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18. Stoichiometry of binding (Fig. 2): The stoichiometry of binding was determined by the method of continuous variations. Solutions ([RNG U5] + [DNA A5] = 2.0 μM) containing different molar ratios of RNG U5 and DNA A5 were heated to 90 °C and allowed to cool slowly to 15 °C. The pH was maintained at 7.0 with 10 mM K_2HPO_4 buffer, while the ionic strength (μ) was held constant with 100 mM KCl. The absorbance of each solution at 260 nm (15 °C) was measured with a Cary 100-E2 9802310Z UV–vis spectrometer. DNA oligomer concentrations were determined spectrophotometrically using extinction coefficients provided by the manufacturer (IDT®). The extinction coefficient value of RNA U5 (ε_{260} = 48700 M^{-1} cm^{-1}) was used for that of RNG U5. The reported absorbance is an average of three experiments (±0.003).
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20. Melting studies (Figs. 3,4): Thermal denaturation (T_m) measurements were obtained by observing the absorbance at 260 nm of a solution of oligomers in 1 cm path-length quartz cuvettes as the temperature was raised 0.5 °C/min from 15 to 90 °C. All samples had been previously annealed by cooling from 90 to 5 °C and stored at 5 °C overnight. Samples consisted of 2 μM RNG U5 and 2 μM DNA A5 with 10 mM K_2HPO_4 buffer at pH 7.0 and μ = 0.100 with KCl. Concentrations of RNG U5 and the polyoligonucleotides were determined spectrophotometrically from molar extinction coefficients [8600 M^{-1} cm^{-1} at 268 nm for poly(dA), 9350 M^{-1} cm^{-1} at 260 nm for poly(rU), 7240 M^{-1} cm^{-1} at 268 nm for poly(dC), 10380 M^{-1} cm^{-1} at 268 nm for poly(dG), and 8220 M^{-1} cm^{-1} at the 268 nm for poly(dT)]. For T_m denaturation, hyperchromicity was used. Data were recorded every 1 °C. Samples were covered with mineral oil to prevent evaporation. The reported T_m values are an average of three experiments (±0.2).
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24. Circular dichroism spectra (Fig. 5): CD spectra were obtained on an OLIS RSM circular dichroism spectrophotometer. Samples were held in a 1 cm path-length cuvette and the temperature was maintained at 20 °C. Scans were run from 320 to 200 nm taking measurements every 1 nm. The integration time for each data point was 2 s. Ten scans were made of each sample, then averaged and smoothed using a 15 point exponential fitting algorithm.