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Incorporation of positively charged ribonucleic guanidine linkages into oligodeoxyribonucleotides: Development of potent antisense agents

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Abstract—Oligodeoxynucleic acid (21-mer) containing both negatively charged phosphate and positively charged ribonucleic guanidine linkages (RNG/DNA chimera) have been synthesized. DNA binding characteristics and nuclease resistance of RNG/DNA chimeras have been evaluated. Using the bcr-abl oncogene (cause of chronic myeloid leukemia) as a target, the binding of a 21-mer RNG/DNA chimera that includes six RNG's is more than 103.5 stronger than the binding of 21-mer composed solely of DNA.

The possible therapeutic use of oligonucleotide analogs as effective gene regulatory agents in antisense and antigene approaches has kindled interest in their development.[1–5] Key goals in the design of such agents include increased binding affinity while maintaining sequence specificity, resistance to degradation by cellular nucleases, and improved membrane permeability. Rapid degradation of natural oligonucleotides' phosphodiester backbones by cellular nucleases necessitated the creation of chemically modified oligonucleotides including methylphosphonate,[6,7] methylenemethylimino (MMI),[8] and amide linkages.[9] An alternative approach involves replacing the oligonucleotide backbone entirely, such as in the case of peptide nucleic acid (PNA),[10–12] phosphonic ester nucleic acid (PHONA),[13] and nucleic acid analog peptide (NAAP).[14] The modified backbones of these oligonucleotide analogs render them resistant to nuclease degradation.

To increase the free energy of oligonucleotide duplex formation, the negative charge–charge repulsion in double-stranded DNA and RNA can be minimized or eliminated if the phosphodiester linkages are replaced by uncharged or positively charged linkages. Addressing these design features led us to develop deoxynucleic guanidine (DNG), which is obtained via the replacement of the negative phosphodiester linkages of DNA [–O-(PO_2^-)-O–] by positively charged guanidinium linkages [–NH-C(= NH_2^+)-NH–].[15–17] This replacement alters the electrostatic nature between strands from being repulsive in native DNA duplexes to attractive in DNG·DNA duplexes. These favorable interactions lead to exceptional binding affinities accompanied by excellent specificities, ideal characteristics for a potential antisense agent.[18–21] In addition to these results, the guanidinium linkage is nuclease resistant,[22] and the positively charged backbones may give rise to increased cell membrane permeability via electrostatic attraction of the guanidine moieties to the negative phosphates on 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 ribonucleic guanidine (RNG), a RNA analog of DNG. 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 suceptible to cellular nuclease nor chemical degradation under physiological conditions.

Keywords: Ribonucleic guanidine (RNG); RNG/DNA chimera; Antisense oligonucleotide.
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Preliminary studies involving binding of cationic pentameric ribonucleic guanidine (RNG-U_5) to DNA and RNA have been reported.[23,24] Melting temperature (T_m) and change in circular dichroism (CD) spectra have been employed to measure the stability of RNG-U_5·DNA-A_5 and RNG-U_5·RNA-A_5 duplexes. These data are compared with data for the corresponding unmodified RNA, DNA duplex to determine the advantage of the stability and structure of the duplexes. The order of thermal stability observed was RNG-U_5·DNA-A_5 > RNA-U_5·RNA-A_5 > RNG-U_5·RNA-A_5 > RNA-U_5·DNA-A_5 > DNA-T_5·DNA-A_5.[23,24]

The CD spectra of the RNG-U_5·RNA-A_5 duplex indicate a structure that is different than the reference structures of the unmodified duplexes.[23,24] The CD spectra and molecular dynamics simulation establish that the instability of the RNG·RNA duplex results from the alteration of the natural conformation of RNA upon the formation of a duplex with RNG. Molecular dynamics suggests that the different functional groups of the RNG·RNA backbone lead to differences in backbone flexibility and length. The duplex compensates for these differences by adopting high levels of base-pair propeller, which in turn causes the natural U-O4···A-N6 hydrogen bond to break. The propeller leaves the U-O4 atom in position to hydrogen bond with the A-N6 atom of the previous base-pair. In contrast, these results also demonstrate that the sustained B-type DNA conformation of the RNG·DNA duplex increases the stability of this duplex due to the electrostatic attraction. The affinity of pentameric ribonucleic guanidine (RNG-U_5) to bind to DNA-A_5 is greater than the affinity of DNA-T_5.

The demonstration of a marked preference of RNG for complementary DNA suggests the synthesis of mixed RNG and DNA sequences and a study of the binding of these DNA/RNG chimeras with long complementary sequences of DNA. Such studies may lead to the synthesis of biologically important antisense/antigene agents.

Ider developed oligonucleotides to target the bcr-abl oncogene, which causes chronic myeloid leukemia (CML) and bcr-abl-positive acute lymphoblastic leukemia (ALL).[25] It was demonstrated that anti-bcr-abl oligonucleotides specifically inhibit bcr-abl mRNA expression up to 70% in hematopoietic cell line and primary CML. Substitution of uridine–uridine phosphodiester linkages with uridine–uridine guanidium linkages on the oligonucleotide strands to target the bcr-abl oncogene (DNA/RNG chimeras) would be expected to be the more potent candidate of antisense against bcr-abl oncogene mRNA.

In this paper, we report the synthesis of 21 base-paired RNG/DNA chimeras, having mixed anionic phosphodiester linkages of DNA and cationic guanidinium linkages of RNG based on the oligonucleotide sequence to target bcr-abl oncogene. The hybridization properties of the RNG/DNA chimeras with complementary DNA have been evaluated using spectroscopic techniques. The stability of these RNG/DNA chimeras toward nucleolytic cleavage has also been investigated.

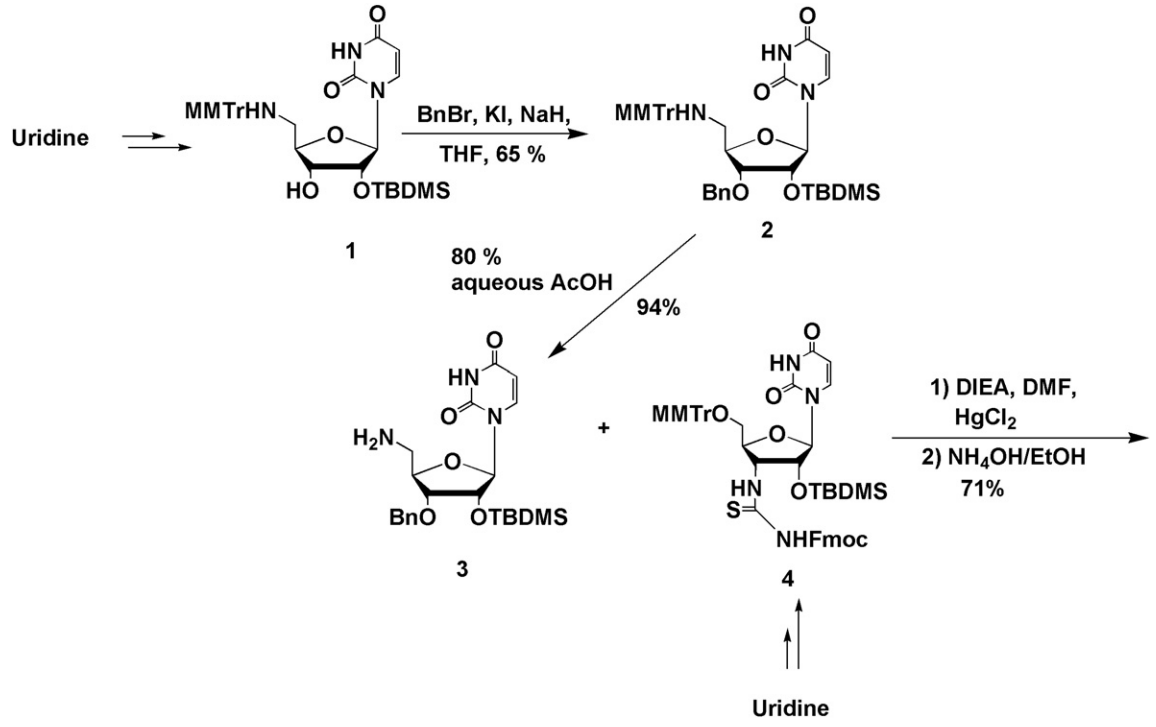
Phosphoramidite **9**[27] was synthesized (Schemes 1 and 2) to facilitate the solid-phase synthesis of oligonucleotide chimeras containing both the standard phosphodiester and the guanidium linkages.[26] Both the required monomers **3** and **4** were prepared from commercially available uridine. The amino uridine derivative **3**[28] was obtained by benzylation[29] of 2′-hydroxy function of **1**[30,31] and subsequent cleavage of the 5′ trityl group of **2**. Coupling of amine **3** with in situ generated carbodiimide from thiourea monomer **4**[30,31] using $HgCl_2/Et_3N$ as reported previously, resulted in Fmoc-protected guanido dimer **5**.[32] Debenzylation of **5** followed by phosphitylation produced phosphoramidite **7**.[33] Finally, Fmoc and silyl groups of **7** were removed to afford the desired phosphoramidite dimer **9**[34] as shown in Scheme 2.

Phosphoramidite **9** was used as a building block to introduce guanidium linkages at desired positions in the chimeric oligonucleotides.[35] The chimeras were synthesized using an automated solid-phase synthesizer with 5′-trityl groups on to allow for HPLC purification. The final detritylated and HPLC purified oligonucleotides were analyzed by mass spectrometry (ESI) and found to be the desired chimeric products (Table 1). Thus, RNG/DNA chimeric oligonucleotides, **10**–**15** having either one (**10**–**13**) or two (**14**–**15**) guanidium linkages were successfully prepared. RNG linkages were introduced at the 3′-terminus (**11**, **13**) and middle positions (**10**, **12**) of chimeras to compare their thermal stability and resistance to nucleolytic cleavage.

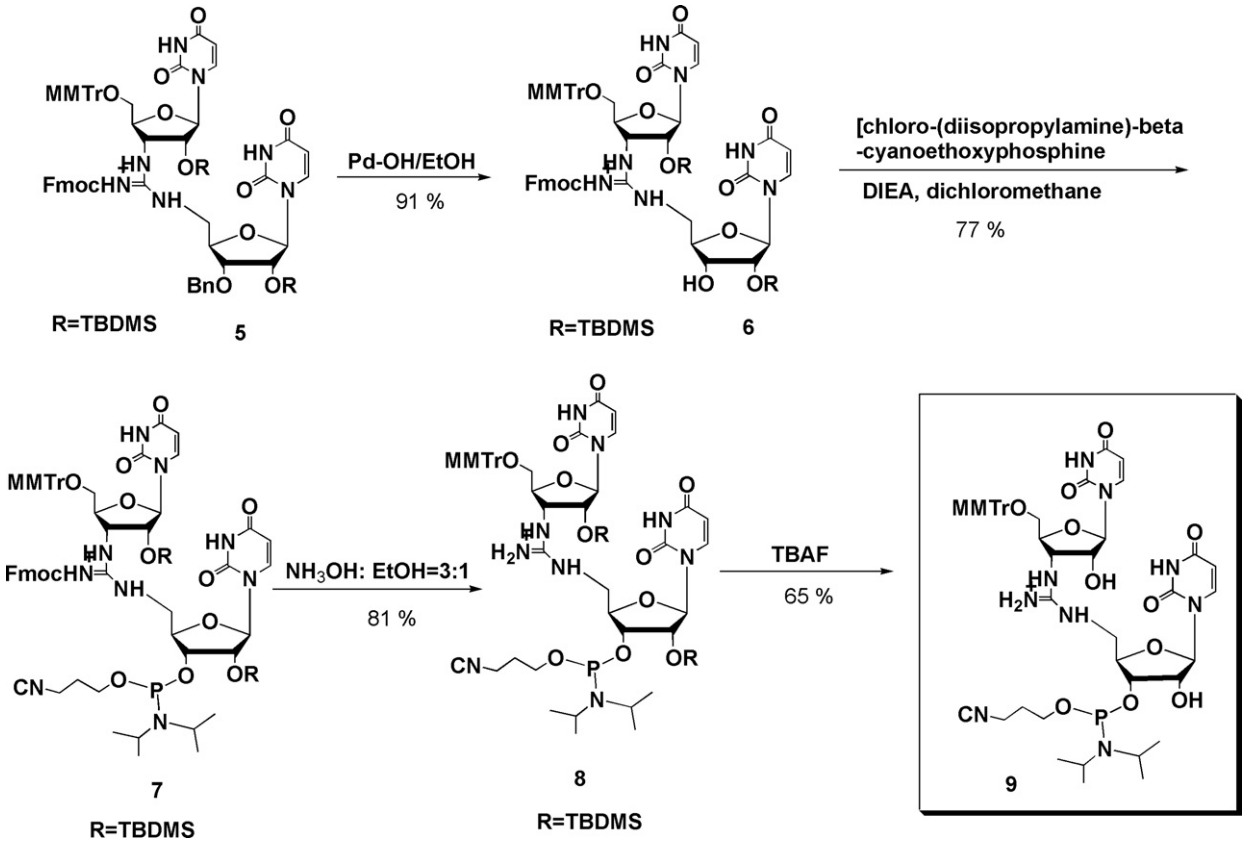
Also, RNG/DNA chimeric oligonucleotides, **19**–**23** (Table 2), having from three to six guanidium linkages were successfully obtained by using synthetic synton **12**[41] in (Scheme 3). Compounds **10**[41] were prepared by a previously reported method.[30,31] Four–six guanidium linkages were introduced as blocking 21-mer DNA by using automated solid-phase DNA synthesizer. The final oligonucleotides were purified by HPLC and analyzed by mass spectrometry. Oligonucleotides **19** and **20** have 4 guanidium linkages but in different positions. Also, oligonucleotides **22** and **23** have 6 guanidium linkages but in different positions (Table 2). In general, the positively charged peptide–oligonucleotide conjugates often show the undesired aggregation formation causing in difficulties for handling. However, there is no trace of self aggregation in the RNG/DNA chimeras.[22b]

All chimeric oligonucleotides were hybridized with their respective complementary strands in a 1:1 mol ratio in phosphate buffer (10 mM Na_2HPO_4, pH 7.1, 0/10/100 mM NaCl). UV-thermal denaturation studies were performed to observe the effect of the guanidium linkage on the formation of duplexes.

All of the temperature versus absorbance curves were sigmoidal (Fig. 1) showing that the double helix formation is cooperative. For the duplex formed with RNG/DNA chimeras (**1**–**6**) and complementary DNA, no apparent difference in T_m occurs between the control



Scheme 1. Preparation of dimer synthon.



Scheme 2. Preparation of dimer synthon 9.

DNA–DNA and RNG/DNA–DNA at all salt concentrations. Furthermore, it is observed that the incorporation of one or two guanidium linkages has no significant effect on the hybridization properties with complementary DNA sequences at all salt concentrations (Table 3).

Table 1. Sequences used for RNG/DNA characterization

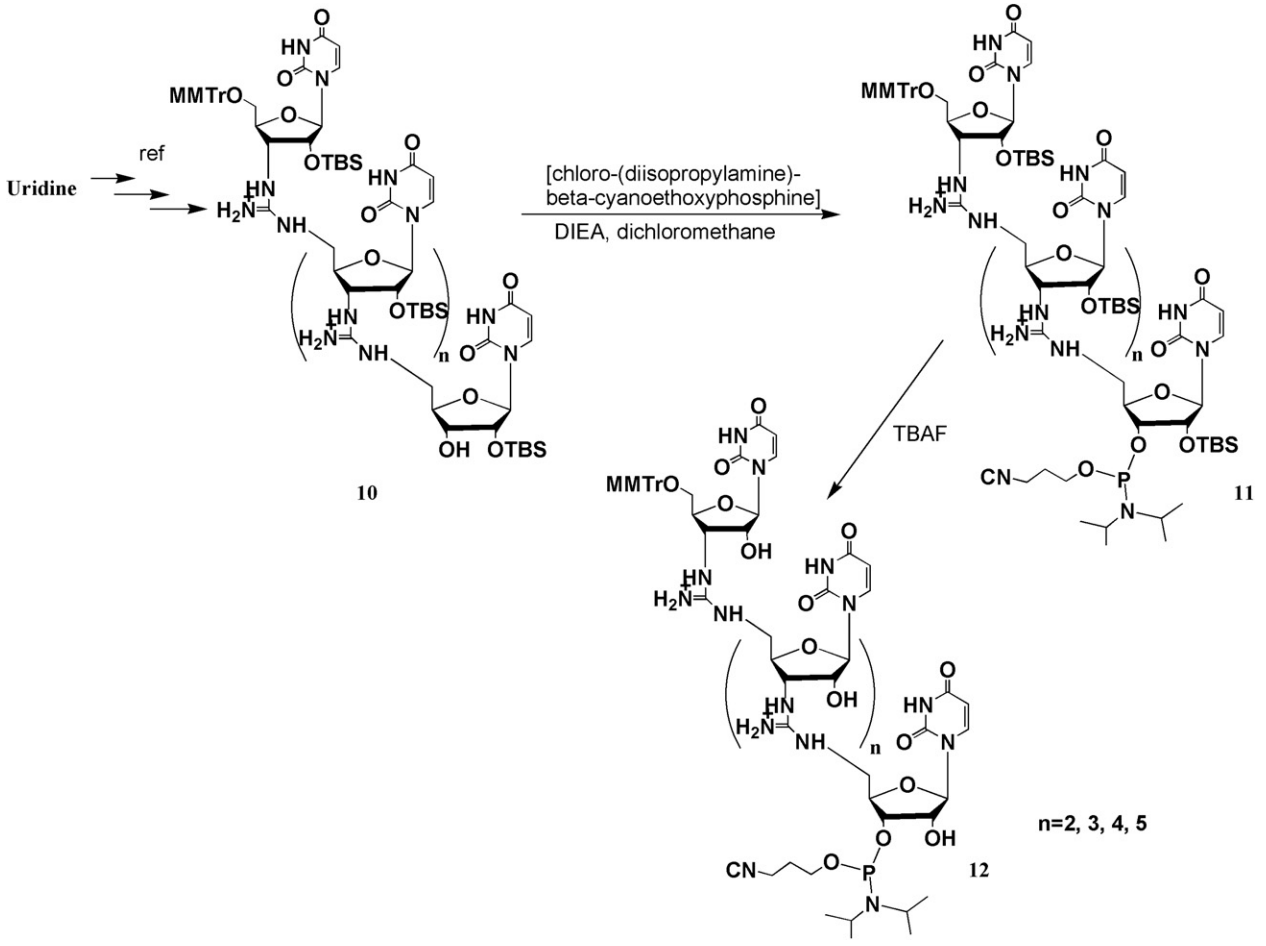
Compound	RNG/DNA chimeras	Yields (%)	Calculated[a] (M)	Founded[b] (*m*/*z*)
13	5′-AAGGGCUgUTTGAACTCTGCTT-3′	42	6588.33	6589.54
14	5′-AAGGGCTTTTGAACTCTGCUgU-3′	40	6588.33	6589.61
15	5′-AGGGCUgUTTGAACTCTGCTTT-3′	45	6565.45	6566.70
16	5′-AGGGCTTTTGAACTCTGCTUgU-3′	43	6565.45	6566.61
17	5′-AAGGGCUgUUgUGAACTCTGCTT-3′	31	6533.40	6534.50
18	5′-AGGGCUgUUgUGAACTCTGCTTT-3′	29	6510.52	6511.74

Oligonucleotides RNG/DNA chimera with one or two guanidium linkages guanidinium linkages indicated as ‘g’.
[a] Calculated molecular weight (M) of oligonucleotides.
[b] Determined molecular weight $(M+H)^+$ of oligonucleotides by mass spectrometry spectra.

Table 2. Oligonucleotides RNG/DNA chimera with four–six guanidium linkages guanidinium linkages indicated as ‘g’

Compound	RNG/DNA chimeras	Yields (%)	Calculated[a] (M)	Founded[b] (*m*/*z*)
19	5′-AGGGTUgUgUgUgUTAACTCTGCTT-3′	28	6311.18	6311.98
20	5′-AGGGTUgUgUgUTTAACTCTGCUgU-3′	26	6313.16	6313.61
21	5′-AGGGUgUgUgUgUgUTAACTCTGCTT-3′	22	6292.24	6292.91
22	5′-AGGGUgUgUgUgUgUgUAACTCTGCTT-3′	19	6273.33	6273.98
23	5′-AGGGUgUgUgUgUgUTAACTCTGCUgU-3′	21	6275.31	6275.76
24	5′-AGGGTTTTTTTAACTCTGCTT-3′		Control	

[a] Calculated molecular weight (M) of oligonucleotides.
[b] Determined molecular weight $(M+H)^+$ of oligonucleotides by mass spectrometry spectra.



Scheme 3. Preparation of synthons 12.

The stability of the RNG/DNA–DNA duplexes increases with the increase in salt concentration (0–100 mM NaCl), as is seen in DNA–DNA duplexes. This indicates that the incorporation of one or two guanidium linkages in DNA does not affect the overall electrostatics of duplex formation with complementary DNA strand.

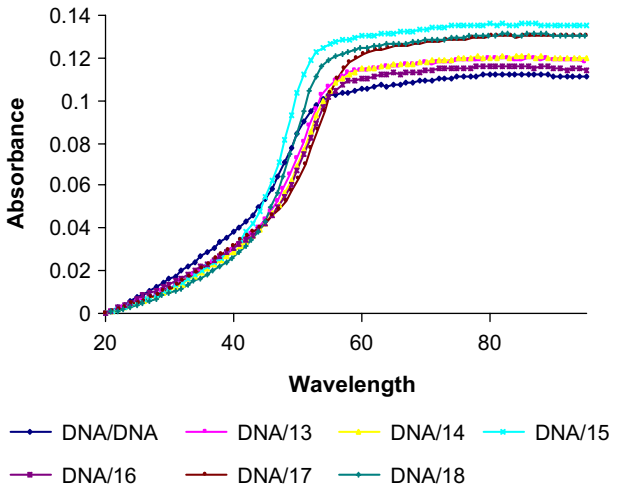


Figure 1. Melting studies 1; thermal denaturation curves for RNG/DNA chimeras and complementary DNA duplexes. Absorbance was measured at 260 nm; the concentration of each strand was 6 μM in 10 mM Na_2HPO_4, 100 mM NaCl, pH 7.1.

The ΔG^0 values (at 25 °C) calculated using the van't Hoff enthalpy values for transitions[36,37] involving RNG/DNA chimera–DNA and DNA–DNA duplexes, showed that the free energy for the formation of RNG/DNA–DNA duplex is about the same as for DNA–DNA duplex. This supports the idea that little structural difference exists between DNA containing one or two guanidium linkages and DNA composed entirely of phosphodiester linkages.

Exonuclease I digests single-stranded DNA by catalyzing the hydrolysis of the phosphodiester linkages from the 3′ to 5′-terminus.[39,40] Thus, it is assumed that upon modification of the phosphodiester linkage at the 3′-terminus, the oligonucleotide could be resistant to exonuclease digestion. To investigate this, RNG/DNA oligonucleotides **1**–**6** were subjected to nucleolytic cleavage by exonuclease I and the hydrolyzate was analyzed by RP-HPLC.[38] Natural unmodified oligonucleotides of same sequences were used as controls.

Under the conditions employed, the control oligonucleotides were readily hydrolyzed to shorter length products within 1 h of incubation; however, the RNG/DNA chimeras **2** and **5** were unaltered even after 12 h of incubation. The RNG/DNA chimeras **1**, **3**, **4**, **6** (rt; retention time = 24.3, 25.0, 24.9, 25.6), which contain one or two guanidinium linkages at the center of the oligonucleotide, was partially hydrolyzed after 1 h (rt = 21.3 and 24.3, 20.3 and 25.0, 22.2 and 24.9, 23.2 and 25.6) of incubation and remained further unaltered even after 12 h. These observations clearly indicate that the RNG/DNA oligonucleotides with positively charged guanidium linkages at 3′-terminus, **2** and **5**, are totally stable to cleavage by nucleolytic enzyme exonuclease I.

The melting temperatures and thermodynamic parameters of chimeras (**19**–**23**) are provided in Figure 2 and Table 3. All experimental conditions employed in melting studies of DNA/RNG chimeras **19**–**23** were exactly the same as those of melting studies of DNA/RNG chimeras with one or two guanidium linkages.

As expected, chimeras **19** and **20** with four charged guanidium linkages in 21-mer DNA binds to complementary DNA with much higher affinity (T_m = 65.0, 65.1 °C) than DNA (48 °C) (Table 3). Also, chimeras **21**–**23**, where five or six phosphodiester linkages have been replaced by guanidium linkages in 21-mer DNA/RNG, bind to complementary DNA with unprecedented higher affinity (T_m = 78.7, 82.4, 83.0 °C). As shown in Table 2, chimeras **19** and **20**, or chimeras **22** and **23** have the same number of guanidium linkages but in different positions. The 3′-end phosphodiester linkage has been substituted with guanidium linkage in chimeras **20** and **23**.

Table 3. Melting temperatures and thermodynamic parameters for helix–coil transitions of RNG/DNA chimeras with complementary DNA

RNG/DNA chimera	Duplex[a]	0 mM NaCl		10 mM NaCl		100 mM NaCl	
		T_m[b] (°C)	$-\Delta G^0_{25}$ kJ/mol	T_m[b] (°C)	$-\Delta G^0_{25}$ kJ/mol	T_m[b] (°C)	$-\Delta G^0_{25}$ kJ/mol
13	**25**	33.1	35.9	38.3	41.5	49.0	53.1
14	**26**	32.9	35.6	37.7	40.8	49.5	53.6
15	**27**	31.8	34.5	37.3	40.4	48.6	52.7
16	**28**	32.6	35.3	38.5	41.7	50.1	54.3
17	**29**	33.3	36.1	38.0	41.2	51.3	55.6
18	**30**	33.1	35.9	38.6	41.8	49.6	53.7
	Control	32.7	35.4	37.0	40.1	48.0	52.0

		100 mM NaCl	
		T_m[b] (°C)	$-\Delta G^0_{25}$ kJ/mol
19	**31**	65.0	60.3
20	**32**	65.1	60,3
21	**33**	78.5	63.0
22	**34**	82.4	72.1
23	**35**	83.0	72.2
	Control	46	51.6

Thermodynamic parameters were calculated by the method of Gralla and Crothers.[36,37]

[a] Absorbance was measured at 260 nm in phosphate buffer; the concentration of each strand was 6 μM.

[b] The reported T_m values are an average of three experiments (±0.2).[38]

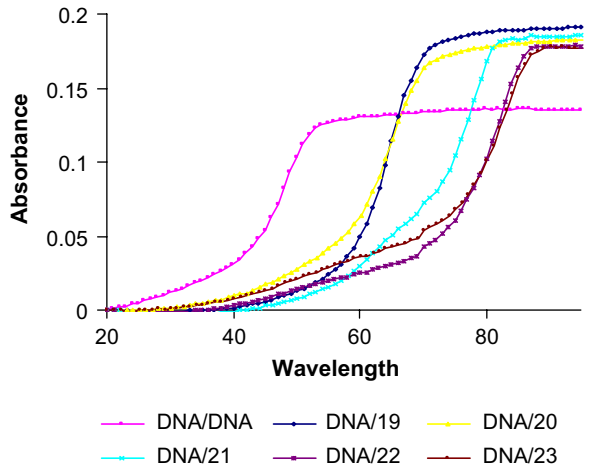


Figure 2. Melting studies 2; thermal denaturation curves for RNG/DNA chimeras and complementary DNA duplexes. Absorbance was measured at 260 nm; the concentration of each strand was 6 μM in 10 mM Na_2HPO_4, 100 mM NaCl, pH 7.1.

Thermodynamic calculations were performed in order to describe the duplex properties more quantitatively. Standard free energies (ΔG^0) for duplex formation are presented in the following equations:

$$\mathrm{DNA(control)} + \mathrm{DNA} \overset{\Delta G^0 = -51.6\ \mathrm{kJ/mol}}{\rightleftharpoons} DNA \cdot DNA \quad (1)$$

$$\mathrm{RNG/DNAchimera},\ \mathbf{20} + \mathrm{DNA} \overset{\Delta G^0 = -60.3\ \mathrm{kJ/mol}}{\rightleftharpoons} \mathbf{20} \cdot \mathrm{DNA} \quad (2)$$

$$\mathrm{RNG/DNAchimera},\ \mathbf{21} + \mathrm{DNA} \overset{\Delta G^0 = -63.0\ \mathrm{kJ/mol}}{\rightleftharpoons} \mathbf{21} \cdot \mathrm{DNA} \quad (3)$$

$$\mathrm{RNG/DNAchimera},\ \mathbf{23} + \mathrm{DNA} \overset{\Delta G^0 = -72.2\ \mathrm{kJ/mol}}{\rightleftharpoons} \mathbf{23} \cdot \mathrm{DNA} \quad (4)$$

The $\Delta\Delta G^0$ of −11.4 kJ/mol (Eq. 3 ΔG°–Eq. 1 ΔG^0) and −20.6 kJ/mol (Eq. 4 ΔG^0–Eq. 1 ΔG^0) translates into an increase in binding over multi orders of magnitude. Results clearly demonstrate the over $10^{3.5}$ tighter binding of RNG/DNA chimeras **22**, **23** to its DNA templates compared to natural DNA duplex. The tremendous increase in the free energy of binding of DNA/RNG chimeras **19**–**23** (Table 3) support the powerful attraction between the positively charged RNG guanidium groups and the negatively charged phosphates of DNA. This increase is attributed to both the formation of hydrogen bonds[37b] in combination with electrostatic attraction of RNG for DNA. This suggests that strategically substituted guanidium-linked uridines may yield powerful antisense oligonucleotide.

A method for the incorporation of a positively charged ribointernucleoside into negatively charged DNA has been demonstrated. The insertion of the guanidium linkage was accomplished using the standard DNA phosphoramidite chemistry and automated solid-phase synthesis techniques. Substitution of one or two positively charged RNG into a 21 DNA sequence provides chimeras that bind to a complementary DNA strand with similar (or a little higher) stability is found for the like DNA/DNA binding. This is a true at various ionic strengths. Incorporation of positively charged guanidium linkages at the 3′-terminus of the DNA prevents digestion by exonuclease I.

The results of melting temperatures of RNG/DNA chimeras in which four–six phosphodiester linkages are replaced by guanidium linkages, show that four guanidium linkages in 21-mer DNA are long enough to enforce the affinity with complementary DNA. Furthermore, oligonucleotide chimeras which have five or six guanidium linkage in 21-mer have tremendously higher T_m temperature compared to natural DNA duplex. It has been strongly suggested that thermodynamically favorable DNA/RNG chimeras may serve as potent antisense candidates. In vitro experiments to evaluate the antisense effect of RNG/DNA chimeras will follow.

Acknowledgment

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27. *Compound **9**:* Compound **8** (294 mg, 0.24 mmol) was dissolved in 1.0 M TBAF in THF (0.5 ml, 0.5 mmol) and stirred at room temperature for 16 h. Acetic acid (0.3 ml) was added, diluted with H_2O, and then Et_2O was added. The aqueous phase was concentrated in vacuo. The residue was purified by RP-HPLC (Altech Macrosphere RP C8 column 0–60% acetonitrile in 3% aqueous acetic acid) (195 mg, 81%). *m/z* (FAB) 999.0 calculated for $C_{49}H_{61}N_9O_{12}P$ $(M+H)^+$ 999.03.
28. *Compound **3**:* Compound **2** (60 mg, 0.1 mmol) was dissolved in 80% aqueous acetic acid and stirred at room temperature for 24 h. The solvent was removed in vacuo. Extraction with ethyl acetate followed by dilution with water, brine, drying over anhydrous Na_2SO_4 and evaporating under reduced pressure. The crude residue was subjected to silica gel column chromatography using dichloromethane/methanol (50:1–1:2) solvent system to give the pure product as a brittle white form (32 mg, 80%). *m/z* (FAB) 448.71 calculated for $C_{49}H_{61}N_9O_{12}P$ $(M+H)^+$ 448.63.
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32. *Compound **5**:* To a mixture of compound **3** (50 mg, 0.12 mmol) and **4** (102 mg, 0.12 mmol) in DMF were added DIEA (0.3 mmol) and $HgCl_2$ (41 mg, 0.15 mmol) for 3 h. The mixture was filtered through a Celite pad. The filtrate was diluted with ethyl acetate, which was diluted with water, brine, dried over anhydrous Na_2SO_4 and concentrated in vacuo. The crude residue was purified by silica gel column chromatography (AcOEt/hexane 1:2, 113 mg, 71%). *m/z* (FAB) 1325.81 calculated for $C_{49}H_{61}N_9O_{12}P$ $(M+H)^+$ 1325.67.
33. *Compound **6**:* A mixture of 5 (60 mg, 0.05 mmol) and 10% Pd/C (10 mg) in EtOH was stirred under 50 psi by parr hydrogenation for 4 h. The catalyst was removed through Celite pad and the filtrate was concentrated in vacuo. The residue was purified by silica gel column chromatography using dichloromethane/methanol (5:1–1:2) solvent system to give the pure product as a brittle white form (56 mg, 91%). *m/z* (FAB) 1235.2 calculated for $C_{66}H_{80}N_7O_{13}Si_2$ $(M+H)^+$ 1235.55.
Compound 7: To a mixture of compound **6** (124 mg, 0.1 mmol) and DIEA (0.15 mmol) in dried dichloromethane was added [chloro-(diisopropylamine)-β-cyanoethoxyphosphine] (0.12 mmol) and stirred for 2 h. The solvent was removed in vacuo. Extraction with ethyl acetate followed by dilution with water, brine, drying over anhydrous Na_2SO_4 and evaporating under reduced pressure. The crude residue was subjected to column chromatography using AcOEt/hexane (1:5–1:2) solvent system to give the pure product as a bright yellow form (112 mg, 77%). *m/z* (FAB) 1449.6 calculated for $C_{76}H_{99}N_7O_{14}PSi_2$ $(M+H)^+$ 1449.80.
34. *Compound **8**:* Compound **7** (145 mg, 0.1 mmol) was dissolved in 28% NH_4OH/EtOH (3:1) and kept at room temperature for 12 h. The mixture was filtered through the glass filter. The filtrate was diluted with ethyl acetate, which was diluted with water, brine, dried over anhydrous Na_2SO_4 and concentrated in vacuo. The crude residue was purified by silica gel column chromatography (AcOEt/hexane 1:2–1:1) solvent system to give the pure product as a bright yellow form (99 mg, 81%). *m/z* (FAB) 1227.2 calculated for $C_{61}H_{89}N_9O_{12}PSi_2$ $(M+H)^+$ 1227.56.
35. *Oligonucleotide synthesis and purification:* All modified oligonucleotides were synthesized on 1.3 μmol scale on Pharmacia Gene Assembler Plus DNA synthesizer. Standard DNA synthesis condition was employed, viz., CPG support and base protected 5′-*O*-(4,4′-dimethoxytrityl)deoxyribonucleoside-3′-[*O*-(diisopropylamino)-β-cyanoethylphosramidite] monomers. The phosphoramidite activated oligomer 9.12 was used at an extra monomer position, in order to introduce a guanidium linkage into Oligonucleotides. The standard synthesis cycle was modified to perform an extended coupling (15 min) during the coupling the modified phosphoramidite oligomer 9.12; a coupling efficiency of >95% was observed for this step.
36. (a) Marky, L. A.; Breslauer, K. J. *Biopolymers* **1987**, *26*, 2601; (b) Breslauer, K. J. In *Methods in Molecular biology*; Agrawal, S., Ed.; Humana: Totowa, 1995; Vol. 26, p 347.
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38. Johnson, W. T.; Zhang, P.; Bergstrom, D. E. *Nucleic Acids Res.* **1997**, *25*, 559.
39. *Exonuclease I digestion studies:* Oligonucleotides 1–6, with phosphodiester or guanidium linkages were treated with exonuclease I (USB). A typical reaction mixture contained the following in 200 μl: 67 mM Tris (pH 8.5), 6.7 mM $MgCl_2$. 20 mM 2-mercaptoethanol, ~0.2 OD of oligonucleotide, and ~20 U of exonuclease I. The reactions were incubated at 37 °C. Aliquots (40 μl) were taken at different time intervals (0.1, 3, 6 and 12 h), quenched by rapid freezing in dry ice-2-propanol bath, and stored frozen until HPLC analysis. Reaction mixtures were analyzed on C-8 RP-HPLC column (Altech, 7 μ, 4.6× 250 mm) using a gradient of 0.5%/mim of CH_3CN in 0.1 M TEAA, pH 7.0, for 50 min at a flow rate of 1 mL/min. Reactions without enzyme were run

for each oligonucleotide and analyzed by HPLC as controls.
40. Hemavathi, C.; Bruice, T. C. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 2423.
41. To a mixture of oligonucleotides 10 (0.1 mmol) and DIEA (0.15 mmol) in dried dichloromethane was added [chloro-(diisopropylamine)-β-cyanoethoxyphosphine] (0.12 mmol) and stirred for 2 h. The reaction solvent was removed in vacuo. Extraction with ethyl acetate followed by dilution with water, brine, drying over anhydrous Na_2SO_4 and evaporating under reduced pressure. The crude oligonucleotide was dissolved in 1.0 M TBAF in THF (0.3 mmol) and stirred at room temperature for 16 h. Acetic acid (0.3 ml) was added, diluted with H_2O, and Et_2O was added. The aqueous phase was concentrated in vacuo. The residue was purified by RP-HPLC (Altech Macrosphere RP C8 column 0–40% acetonitrile in 3% aqueous acetic acid). Mass spectrometry data for compound **10**, **11**, **12**.

n	**3**		**4**		**5**		**6**	
Compound	*m/z* (FAB)	Calculated for $(M+H)^+$	*m/z* (FAB)	Calculated for $(M+H)^+$	*m/z* (FAB)	Calculated for $(M+H)^+$	*m/z* (FAB)	Calculated for $(M+H)^+$
10	1871.01	$C_{89}H_{146}N_{17}O_{19}PSi_4$ 1871.51	2283.25	$C_{107}H_{181}N_{22}O_{23}PS_5$ 2283.26	2696.73	$C_{125}H_{216}N_{27}O_{27}PS_6$ 2696.50	3111.75	$C_{143}H_{251}N_{32}O_{31}PS_7$ 3111.32
11	2.71.01	$C_{98}H_{163}N_{19}O_{20}PSi_4$ 2070.11	2484.37	$C_{116}H_{198}N_{24}O_{24}PS_5$ 2484.36	2897.95	$C_{134}H_{233}N_{29}O_{28}PS_6$ 2898.61	3311.5	$C_{152}H_{268}N_{34}O_{32}PS_7$ 3311.8
12	1613.77	$C_{74}H_{107}N_{19}O_{20}P$ 1613.73	1911.93	$C_{86}H_{128}N_{24}O_{24}P$ 1911.92	2212.09	$C_{98}H_{149}N_{29}O_{28}P$ 2212.38	2511.75	$C_{110}H_{170}N_{34}O_{322}P$ 2511.25