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Development of potential anticancer agents that target the telomere sequence

Myunji Park, Thomas C. Bruice *

Department of Chemistry and Biochemistry, University of Califonia at Santa Barbara, CA 93106, USA

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ABSTRACT

The immortality of cancer cells is due to the relatively high concentration of telomerase enzyme that maintains the telomere sequence during cell division. Human telomeric DNA consists of repeats of the sequence d(5′-TTAGGG-3′). Deoxyribonucleic guanidine (DNG) is a DNA analog in which positively charged guanidine [–NH–C(=NH_2^+)–NH–] replaces the negatively charged phosphodiester of DNA. The synthesized DNG hexamer AgAgTgCgCpC and dodecamer AgAgTgCgCgCAgAgTgCgCpC are complementary to the non-coding telomere sequence d(5′-TTAGGG-3′). We have found that binding of the complementary DNG hexamer to the telomere is favored over that of DNA telomere by $10^{2.5}$-fold and binding the dodecamer with 2-mismatched DNA is favored by 10^5-fold. We have shown that DNG binding to RNA is favored over binding to DNA. A complementary complex of DNG with RNA at the active site of telomerase enzyme would be very stable.

To prevent the loss or mutation of essential genes, the ending of each chromosome is capped by a special strand of DNA called a telomere. Without telomere caps, chromosomes would be open to a wide range of catastrophic events preventing the DNA molecule from replicating. Telomeres, therefore, play an important role in maintaining the integrity of DNA in human cells. It is thought that telomeres at the end of chromosome may have evolved to prevent the unlimited growth of cells by limiting their life span. Evidence that the average length of telomere correlates with the lifespan of the cell in culture, suggests that the growth of human cells is limited by their resource of telomeric DNA.[1]

Human telomeric DNA consists of repeats of the sequence d(5′-TTAGGG-3′) on one strand and a complementary sequence on the matching strand (3′-AATCCC-5′).[2–4] The telomere single strands serve as substrate for telomerase enzyme, which rebuilds the telomere strand lost during cell division. The telomerase enzyme consists of a reverse transcriptase protein with an 11-base RNA template (5′-CUAACCCUAAC-3′) at the active site.[5] The telomerase synthesis of telomere is regulated by telomere-specific proteins such as protection of telomerase-1 (POT1).[6] A model, provided by Lei et al., demonstrated that in cell division the length of the 3′-end overhanging telomere single strand form POT1 is crucial to the synthesis of telomere sequences.[7] Telomerase catalysis is inactive when the overhanging telomere single strand consists of fewer than six nucleotides while it is highly active when the single strand contains more than eight nucleotides.

In adult somatic cells, the mechanisms for maintaining the length of the telomere are absent. Thus, with each successive round of cell division, telomeres progressively decrease in length, loosing up to 200 base pair of terminal DNA from the tips of chromosomes with each division. Since each chromosome possesses a telomere of finite length, the number of divisions a cell can undergo is limited. The more times a cell divides, the shorter the telomere becomes and ultimately it becomes so short that cell ceases to divide. This non-dividing state is known as senescence. Cancer cells, in contrast, maintain a steady population of telomerase, and this makes them immortal.[1]

Two possible approaches to prevent telomere replacement in cancerous cells are: (i) altering the overhanging telomere single strand so that it is no longer a substrate for the telomerase enzyme; (ii) inhibition of the telomerase enzyme by blocking the telomeric RNA 11-base template.

Telomerase inhibition is a well known approach in cancer therapeutics.[5] The DNA substrate of telomerase is a polyanion. Drugs invented as inhibitors of enzymes operating on DNA are also generally polyanions. An example is GRN163L (5′-pam-TAGGGTTAGACAA-NH_2-3′), a lipid conjugated 13-mer thiophosphoramite oligomer (Geron Corporation, California). GRN163L is reported to inhibit human telomerase and has clearance by the US FDA to enter human phase I/II clinical testing against chronic lymphocytic leukemia and breast cancer.[8]

We have reported the synthesis and binding properties of deoxynucleic guanidine (DNG), wherein the negatively charged phosphodiester linkages of DNA have been entirely replaced by positively charged guanidium linkages[–NH–C(=NH_2^+)–NH–].[13,14] Since DNA is a polyanion, a polycationic complementary nucleotide sequence would provide a strong and useful drug. DNG and RNG compounds were invented in our laboratory.[9–11] Polyanion DNA binds to polycation DNG with high affinity and specificity (Fig. 1). Moreover,

* Corresponding author. Tel.: +1 805 893 2044; fax: +1 805 893 2229.
E-mail address: tcbruice@chem.ucsb.edu (T.C. Bruice).

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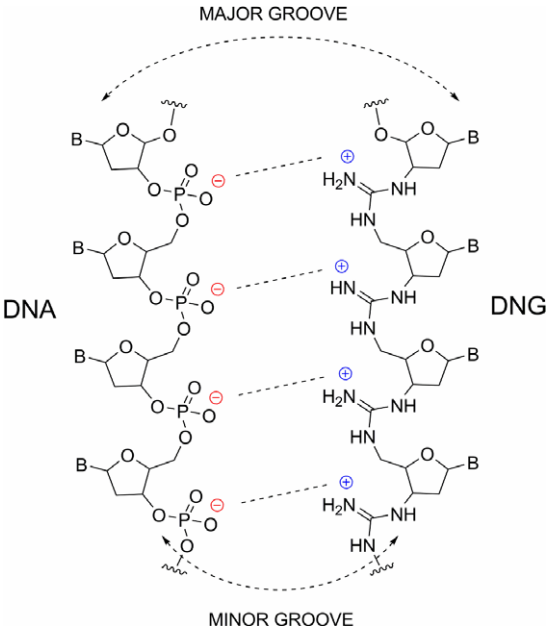


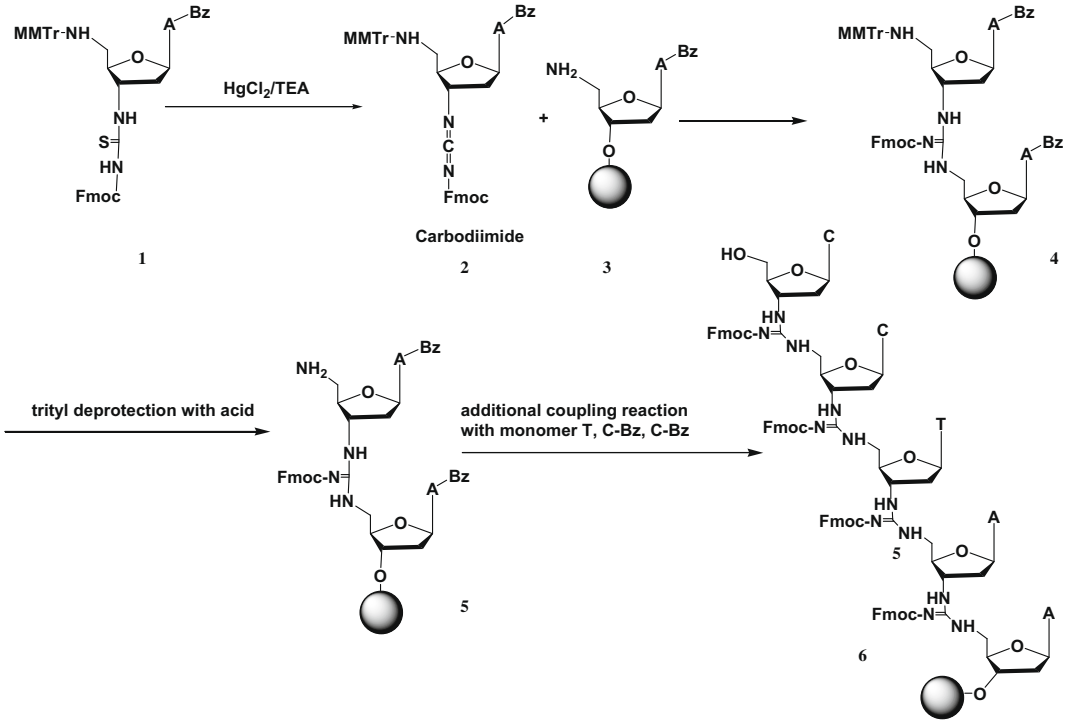
Figure 1. DNA·DNG complex showing the electrostatic attraction between negatively charged phosphodiester linkages and positively charged guanidinium linkages.

DNA intervenes less with telomere-specific proteins due to the positively charged binding sites of these proteins.[12]

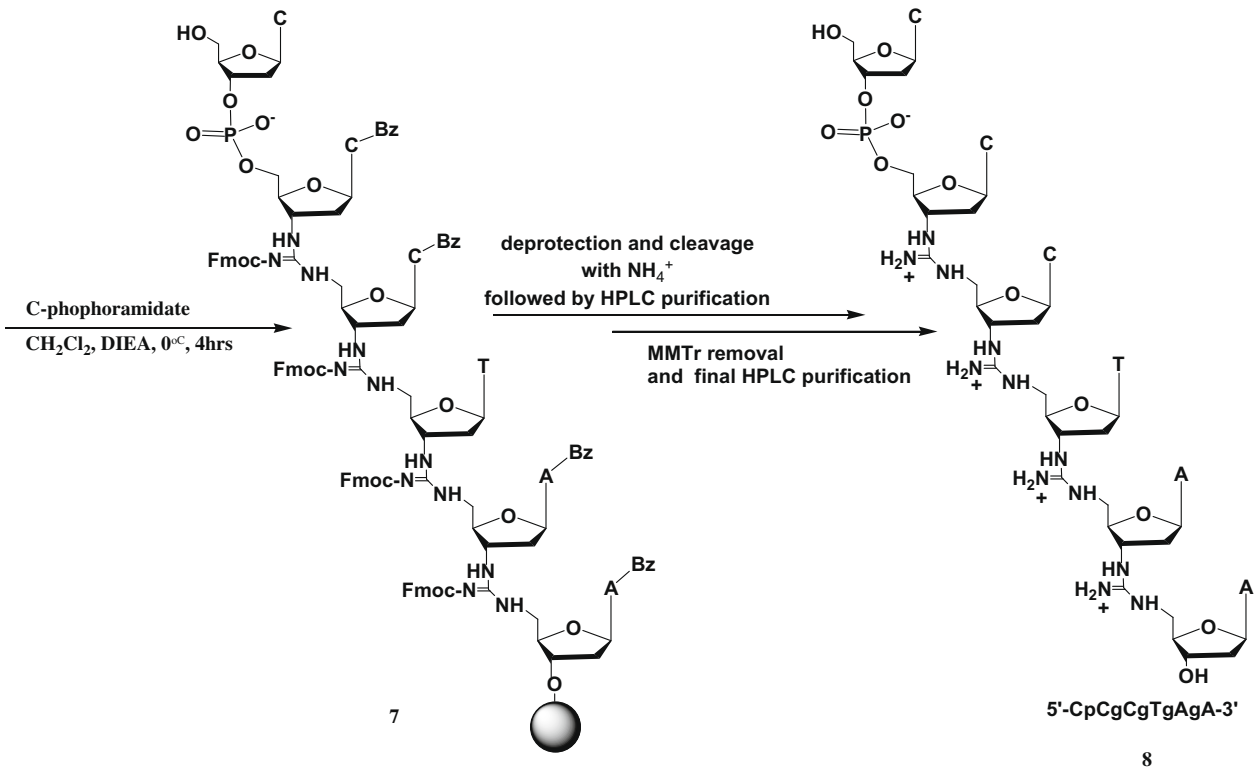
DNG binds strongly to target DNA because the repulsive electrostatic interactions of duplex DNA are replaced by close attractive electrostatic interactions in the DNA·DNG duplex (Fig. 1). From computational studies,[10,15,16] DNG is anticipated to maintain its positive charge in proper alignment to maximize its interaction with the backbone of the negatively charged phosphodiester linkage of the opposite strand. A pentameric thymidyl oligomer of DNG has been shown to bind to polyadenine DNA with unprecedented high affinity in a 2:1 thymine–adenine complex.[10] Interaction with polyguanine, polycytidine, and polythymine were not observed; DNG exhibits fidelity of base pair recognition. In addition, octameric thymidyl DNG oligomer was able to discriminate between complementary and noncomplementary base pairs. Computational molecular modeling suggests that the DNG·DNA duplex primarily retains a B-DNA conformation, whereas the DNG·RNA duplex adopts an A-type structure.[9] In addition, the guanidium linkage has been shown to be resistent to nuclease hydrolysis.[17] The positively charged backbones of DNG may give rise to cell membrane permeability through electrostatic attraction of the guanidine moiety with the negatively charged phosphate groups on the cell surface. Because of the potential antisense/antigene aspects of DNG, further studies on the synthesis and properties of guanidium linked oligonucleotides are warranted.

In this Letter, we report the synthesis of the guanidium linked complementary unit of telomere sequence, heptamer 3′-AgAgTgCgCpC-5′, dodecamer 3′-AgAgTgCgCgCgAgAgTgCgCpC-5′ and the evaluation of the thermal stabilities of heptamer and dodecamer with complementary DNA telomere.

The synthesis of the guanidium internucleotide linkage involves in situ generation of a carbodiimide **2** from a Fmoc protected thiourea using $HgCl_2/Et_3N$ and its subsequent coupling with amine **3** (A-Bz) as shown in Scheme 1. The all monomer using coupling reaction were prepared by previously reported methods.[14–16] After removing the MMTr protecting group from the 5′-position of **4**, the coupling reaction with liberated amine with incoming coupling monomer (T, C-Bz, C-Bz) provides compound **6**. The coupling reaction has already been presented.[16,17] Oligonucleotide **6** was coupled with the cytidine aminophosphoramidate to facilitate the phosphorous linkage at the 5′-end (Scheme 2).[18] Finally, cleavage from solid support and removal of the protecting groups afforded the desired DNG oligonucleotide**.** The final detritylated and HPLC purified oligonucleotide was analyzed by mass spectrometry



Scheme 1. The synthesis of DNG AgAgTgCgCpC I.



Scheme 2. The synthesis of DNG AgAgTgCgCpC II.

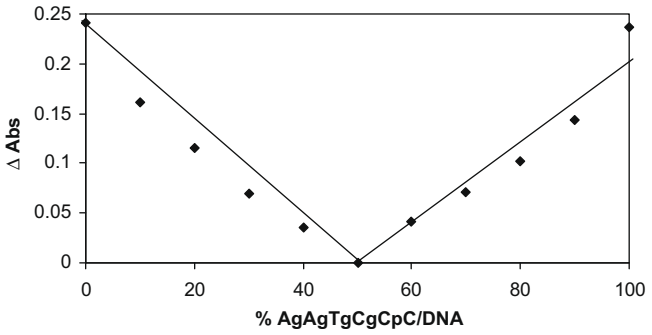


Figure 2. [19]Job plot mixing curves of the change of absorbance at 260 nm (ΔA_{260}) of aqueous solutions (pH 7.0, μ = 0.12 μM at 20 °C) with varied mole fractions of DNG and DNA ([AgAgTgCgCpC] + [TpTpApGpGpG] = 2.0 μM). The inflection point indicates the stoichiometry to be a AgAgTgCgCpC/TpTpApGpGpG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

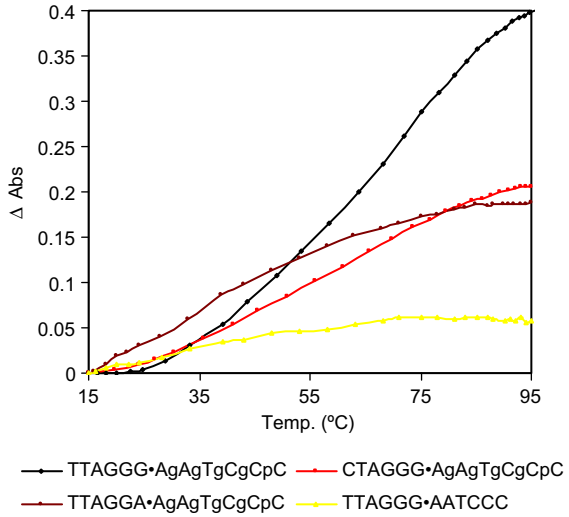


Figure 3. [21]Melting studies; thermal denaturation curves for AgAgTgCgCpC and DNA duplexes. Absorbance was measured at 260 nm; the concentration of each strand was 6 μM in 10 mM Na_2HPO_4, 100 mM NaCl, pH 7.1 (guanidinium linkages were indicated as 'g').

(ESI) and final to be the desired product **8** (yield = 21%, m/z = 1643.65, 1654.45. 1680.81 $(M+H/Na/K)^+$; calculated 1642.73).

The stoichiometry[20] of binding of AgAgTgCgCpC and DNA was determined by the method of continuous variation to generate mixing curves of the absorbance versus mole fraction of AgAgTgCgCpC and DNA (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 the mole fraction of AgAgTgCgCpC compared to the DNA (pH 7.1 and μ = 0.12 μM with KCl at 20 °C) lowered the UV absorbance at 260 nm. An inflection point at 0.5 mole fraction indicated the formation of the AgAgTgCgCpC·TpTpApGpGpG duplex with the expected 1:1 stoichiometry.

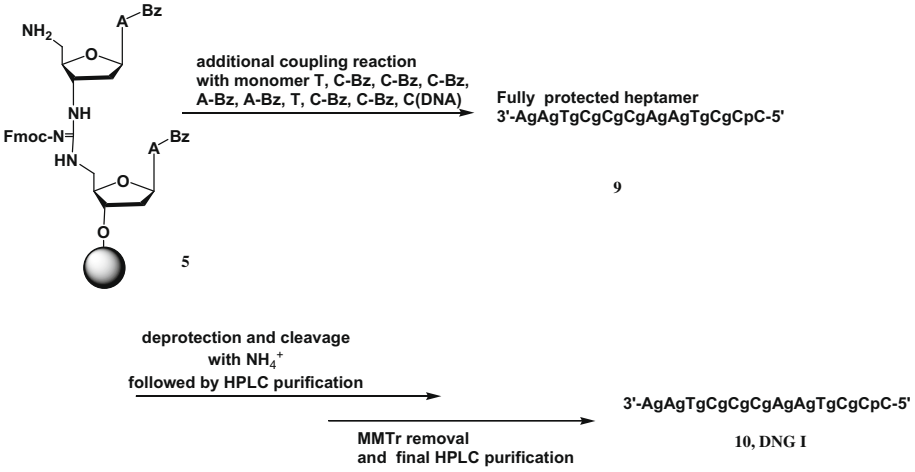
The stability of the duplexes formed between DNG AgAgTgCgCpC and complementary DNA was studied by thermal denaturation experiments (Fig. 3).[21] To confirm the effect of the guanidium linkage of the DNG on the thermal stability of the duplexes, the T_m values for an unmodified DNA·DNA duplex was also determined. The AgAgTgCgCpC with four charged guanidium linkage binds to complementary DNA with much higher affinity than DNA duplex by 3.5 kcal/mol ($\sim 10^{2.5}$-fold).

To study the sequence specificity of the binding of complementary DNA, AgAgTgCgCpC was allowed to form duplexes with complimentary DNA and mismatch DNAs, and the thermal stability of the resultant duplexes were monitored by thermal denaturation (Fig. 3). The duplexes AgAgTgCgCpC·CpTpApGpGpG and AgAgTgCgCpC·TpTpApGpGpG duplexes, contains one mismatched

Table 1
Melting temperatures and thermodynamic parameters for helix-coil transitions of 3′-AgAgTgCgCgCgAgAgTgCgCpC-5′ (**I**) with DNA's at pH 7.1

No	Oligonucleotide	0 mM NaCl		10 mM NaCl		100 mM NaCl	
		T_m[b] (°C)	ΔG_{25}[c] (−kcal/mol)	T_m[b] (°C)	ΔG_{25}[c] (−kcal/mol)	T_m[b] (°C)	ΔG_{25}[c] (−kcal/mol)
1	5′-TTAGGGTTAGGG-3′	ND		ND		ND	
2	5′-<u>A</u>TAGGGTTAGG<u>T</u>-3′	62	10.3	67	10.5	79	14.2
3	5′-<u>A</u>TAGG<u>T</u>TTAGG<u>T</u>-3′	54	9.9	56	10.1	75	12.9
4	5′-<u>A</u>TAGG<u>T</u>T<u>A</u>AGG<u>T</u>-3′	44	9.5	47	9.8	65	10.4
5	5′-<u>AGTAT</u>GTTAGGG-3′	41	9.2	43	9.6	47	9.8
6	5′-TTAGGGT<u>TCAAA</u>-3′	42	9.2	44	9.6	46	9.8
7	Control[a]	—	—	—	—	10[d]	7.1[e]

ND: Not dissociated.
Underlined base pair indicates mismatched one with DNG I.
[a] Control: DNA duplex of 5′-ATAGGGTTAGGT-3′ and 3′-AATCCCAATCCC-5′.
[b] The reported T_m values are an average of three experiments (±0.2).[21] Absorbance was measured at 260 nm in phosphate buffer; the concentration of each strand was 6 μM.
[c] Thermodynamic parameters were calculated by the method of Gralla and Crothers.[23]
[d] Conservative estimate of T_m = 7 °C, which were obtained from extrapolated T_m curve was used for the calculation.
[e] ΔG^o values were calculated from extrapolated melting curve manually as they did not exhibit clear T_m transitions due to the low T_m values. The thermodynamic data for the melting transitions of duplexes were calculated following the work of Marky and Breslauer.[24,25]



Scheme 3. The synthesis of DNG I AgAgTgCgCgCgAgAgTgCgCpC.

base, exhibits huge decrease in T_m in comparison to a fully complementary AgAgTgCgCpC·TpTpApGpGpG duplex.

Besides, dodecamer DNG, AgAgTgCgCgCgAgAgTgCgCpC (I, Table 1) was synthesized (Scheme 3) and thermal stability was evaluated (Fig. 4). The final product (10, DNGI) was purified by HPLC and analyzed by mass spectrometry (ESI) (yield = 9%, m/z = 3335.59, 3347.21, 3373.71 $(M+H/Na/K)^+$; calculated = 3334.43) The melting temperatures and thermodynamic parameters of DNG/DNA are provided in Table 1. The melting temperature of the duplex of **I** with fully complementary DNA does not melt at the temperatures employed (5–95 °C). The binding is too tight to dissociate (No 1, Table 1). DNG I which has 10 guanidium linkages binds to DNA with two mismatched base pair with unprecedented higher affinity (No 2, Table 1) than control (No 7, Table 1).

$$\text{DNA} + \text{DNA} \underset{}{\overset{-\Delta G^0 = 7.1\ \text{kcal/mol}}{\rightleftharpoons}} \text{DNA/DNA} \quad (1)$$

$$\text{DNG } \mathbf{I} + \text{DNA } \mathbf{2} \overset{-\Delta G^0 = 14.2\ \text{kcal/mol}}{\rightleftharpoons} \text{DNG } \mathbf{I} / \text{DNA}\mathbf{2} \quad (2)$$

The $\Delta\Delta G^o$ of −7.1 kcal/mol (Eq. (2)) ΔG^o − (Eq. (1)) ΔG^o) translates into an increase in binding over multi orders of magnitude. Results clearly show to be over 10^5 tighter binding of DNG I to its DNA templates compared to natural DNA duplex. The tremendous increase in the free energy of binding of DNG **I** (Table 1) supports the powerful attraction between the positively charged DNG guanidium groups and the negatively charged phosphates of DNA. This increase is attributed to both the formation of hydrogen bonds in combination with electrostatic attraction of DNG for DNA. Also, that explains why the duplexes DNG/DNA with mismatched sequences (Table 1) have higher melting points than DNA duplex. Our previous researches on DNG·DNA duplexes show that the melting temperatures decrease with increasing ionic strength with KCl. On the other hand, the studies of DNG–DNA chimeras have been demonstrated that melting temperatures have been increased with increasing ionic strength with NaCl as Table 1 shows in this study.[17]

It is interesting to note that DNG demonstrates a marked preference in binding RNA compared to DNA.[22] This suggests that strategically substituted DNG may yield powerful antisense oligonucleotides targeting the RNA template in the telomerase. The study of the binding properties of DNG AgAgTgCgCpC and AgAgTgCgCgCAgAgTgCgCpC with RNA will follow.

From the above discussion, the following conclusions can be drawn about DNG: (i) The DNG complementary unit of telomere, AgAgTgCgCpC and AgAgTgCgCgCAgAgTgCgCpC have been synthesized using the automated solid-phase synthesis. (ii) The DNG AgAgTgCgCpC binds to an complementary DNA strand in a 1:1 ratio. (iii)

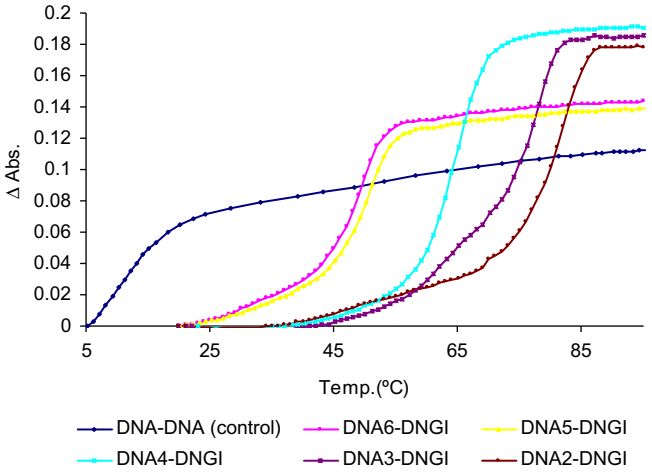


Figure 4. [21]Melting studies; thermal denaturation curves for AgAgTgCgCgCg AgAgTgCgCpC and DNA duplexes. Absorbance was measured at 260 nm; the concentration of each strand was 6 μM. **DNA2**: 5′-ATAGGGTTAGGT-3′, **DNA3**: 5′-ATAGGTTTAGGT-3′, **DNA4**: 5′-ATAGGTTAAGGT-3′, **DNA5**: 5′-AGTATGTTAGGG-3′, **DNA6**: 5′-TTAGGGTTCAAA-3′, control: DNA duplex of 5′-ATAGGGTTAGGT-3′ and 3′-AATCCCAATCCC-5′ (guanidinium linkages were indicated as 'g', underlined base pair indicates mismatched one with DNG I).

The DNG AgAgTgCgCpC was able to discriminate between complementary and noncomplementary base pairs. (iv) Due to electrostatic attraction in place of electrostatic repulsion, a DNG dimeric complex with DNA is most thermodynamically favored over DNA·DNA duplexes. The sequence specificity and thermodynamically favored high affinity of binding of DNG AgAgTgCgCpC and AgAg TgCgCgCAgAgTgCgCpC with complementary DNA lead us to explore and develop new antisense agents targeting telomere. The in vivo experiments will follow.

Acknowledgment

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18. *Synthesis of* **7**: *C*-Phosphoramidate (50 mg) was added to a solution of compound **6** in DMF at 0 °C and stirred at ice bath (0 °C) for 4 h. The solid was collected by washing with dichloromethane and ether and vacuum dried.
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20. *Stoichiometry of binding* (Fig. 1): The stoichiometry of binding was determined by the method of continuous variations. Solutions ([DNG AgAgTgCgCpC] + [DNA] = 2.0 μM) containing different molar ratios of DNG and DNA 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 reported absorbance are an average of three experiments (±0.003)
21. *Melting studies* (Figs. 2 and 3): 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 DNG and 2 μM DNA A5 with 10 mM K_2HPO_4 buffer at pH 7.1. Concentrations of DNG and the polyoligonucleotides were determined spectrophotometrically from molar extinction coefficients [8600 M^{-1} cm^{-1} at 268 nm for poly (dA), 7240 M^{-1} cm^{-1} at 268 nm for poly (dC), 10,380 M^{-1} cm^{-1} at 268 nm for poly (dG), 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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