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Binding properties of positively charged deoxynucleic guanidine (DNG), AgTgAgTgAgT and DNG/DNA chimeras to DNA

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ABSTRACT

The melting properties of hexameric oligonucleotide AgTgAgTgAgT, in which the phosphodiester linkages of the DNA have been replaced by guanidium linkages, have been evaluated. Using the juvenile esterase gene as a target, the binding of a 20-mer DNG/DNA chimera that includes AgTgAgTgAgT is more than $10^{5.7}$ stronger than the binding of 20-mer composed solely of DNA.

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The fungus *candida albicans* infects humans and is particularly pernicious for immunopromised hosts, such as cancer and AIDS patients. Because the number of humans infected by fungi and the occurrence of resistant strains are increasing, finding new compounds to treat this infection is important.[1] In vitro results suggest that oligonucleotides are promising compounds for targeting DNA or RNA in fungal pathogens.[2,3]

The high affinity and specificity of Watson–Crick hybridization have made oligonucleotides attractive agents for diagnostic and therapeutic applications.[4] Oligonucleotides have several advantages for targeting DNA or RNA. Pairing rules between an oligonucleotide and DNA or RNA facilitate rational design. Gene expression can be reduced by RNase H cleavage of target RNA[5] or by blocking transcription and translation.[6] Oligonucleotides can also serve as suicide inhibitors[2,3] or to misfold RNA.[7] Polynucleotides are easily synthesized[8] and this allows rapid testing of oligonucleotides of different composition. Properties such as higher affinity and nuclease stability can be programmed into oligonucleotides by modifying the backbone, sugar, or base.[4] The primary goal of these modifications has been to improve biostability and cellular uptake of the oligonucleotides in order to optimize their tissue and cell distribution for a particular molecular target. The mechanism of action of antisense oligonucleotides requires specific hybridization of the oligonucleotide at its complementary site on the mRNA.

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.[9,10] 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,[10a,11,12] DNG is anticipated to maximize its interaction with DNA by maintaining its positive charge in proper alignment 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 poly-adenine DNA with unprecedented high affinity in a 2:1 thymine–adenine complex.[10a] Since interaction with poly-guanine, cytidine, and thymine was not observed, DNG maintains fidelity of base-pair recognition. 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.[10b] In addition to these results, the guanidium linkage was shown to have nuclease resistance[13] 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 activity of DNG, further studies on the synthesis and properties of guanidium linked oligonucleotides are warranted.

The use of oligonucleotides in cell culture poses several challenges. For example, uptake into mammalian cells is not efficient and oligonucleotides are often degraded after entering cells.[5] Transfection agents, small molecules, and peptides have been used to circumvent these problems in mammalian cells.[14] However, little is known about oligonucleotide uptake into other organisms, such as fungi. Cell membranes and metabolism differ substantially between fungi and mammalian cells, thus uptake and metabolism of oligonucleotides may differ. Disney et al. have reported that oli-

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gonucleotides (hexamer; GCCTCT) are readily taken into *C. albicans* in an energy-dependent manner, producing intracellular concentrations as much as 100-fold higher than the concentrations in the media.[15] They also have shown that hexameric oligonucleotides are not significantly degraded in *C. albicans* after incubations of 12 h and that the intracellular concentration due to oligonucleotide uptake by mammalian cells is at least 10-fold lower than for *C. albicans*. This means that uptake may provide selectivity for targeting fungi with oligonucleotides.

The hexameric oligonucleotide ATATAT is one of most overrepresented of the yeast genomic downstream sequences.[16a] ATATAT is an essential nucleotide involved in regulation of transcription and translation processes in yeast cells. Here, we report the DNA binding properties of the hexameric DNG AgTgAgTgAgT and the synthesis of 20 base paired DNG/DNA chimeras having mixed anionic phosphodiester linkages of DNA and cationic guanidinium linkages of hexameric AgTgAgTgAgT. The structure of the DNG/DNA chimera is based on the oligonucleotide sequence targeting the juvenile hormone esterase gene.[16b] The hybridization properties of the DNG/DNA chimeras with complementary DNA have been evaluated using spectroscopic techniques.

Hexameric oligonucleotide **1**[17] (Fig. 1) was synthesized from commercially available 2′-deoxyadenosine and 2′-deoxythymidine by a previously reported method through several steps.[16] Phosphoramidite **3**[19] was synthesized (Scheme 1) to facilitate the solid-phase synthesis of oligonucleotide chimeras containing both the standard phosphodiester and guanidium linkages.[20] Compound **2**[18] was activated for use in solid-phase synthesis by phosphitylation using [chloro-(diisopropylamino)-β-cyanoethoxyphosphine] to provide the desired phosphoramidate **3**. Phosphoramidite **3** was used as a building block to introduce guanidium linkages at desired positions in the chimeric oligonucleotides. The chimeras were synthesized using an automated solid-phase synthesizer with 5′-trityl groups which allows HPLC purification.[21] The final detritylated and HPLC purified oligonucleotides were analyzed by mass spectrometry (ESI) and found to be the desired chimeric products. As shown in Table 1, 3′-end (**6**), 5′-end (**5**), and 3′, 5′-end (**7**) mismatched chimeras were synthesized to examine sequence specificity.

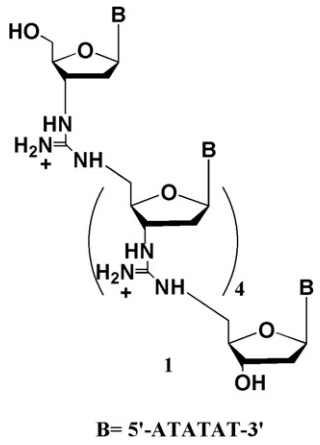
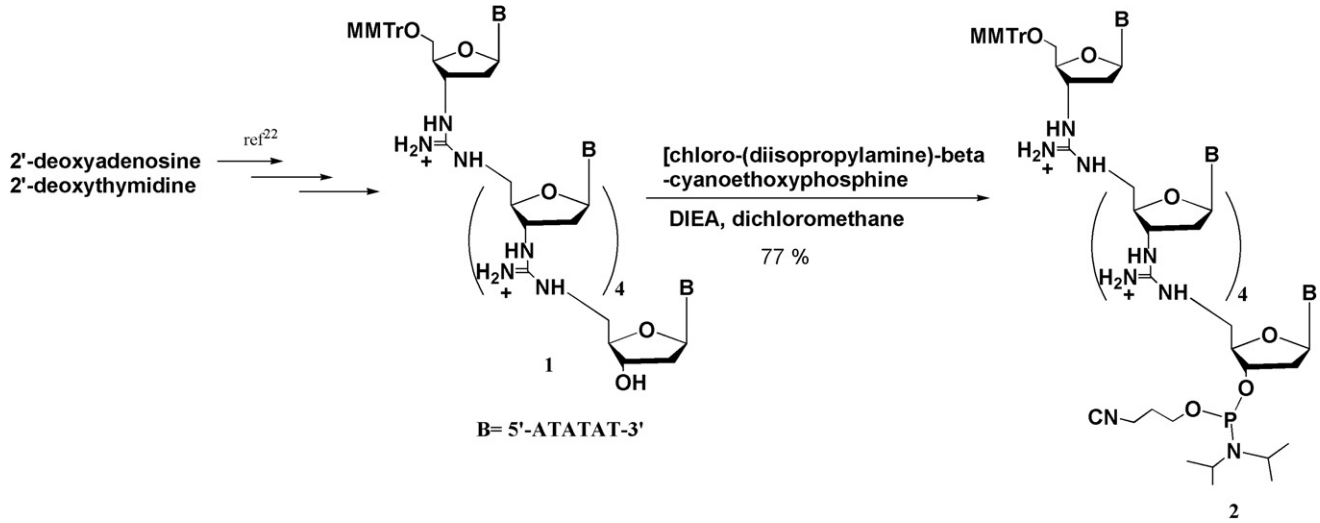


Figure 1. Structure of hexameric DNG, AgTgAgTgAgT ('g' indicated guanidinium linkage).

The binding stoichiometry[22] of AgTgAgTgAgT and DNA was determined by the method of continuous variation[23] to generate mixing curves of the absorbance versus mole fraction of AgTgAgTgAgT 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 mole fraction of AgTgAgTgAgT to the DNA (pH 7.0 and $\mu = 0.12$ with KCl at 20 °C) lowered the UV absorbance at 260 nm. An inflection point at 0.5 mol fraction indicated the formation of AgTgAgTgAgT·DNA duplex with the expected 1:1 stoichiometry.

The stability of the duplexes formed by AgTgAgTgAgT and DNA was studied by thermal denaturation experiments (Fig. 3).[24,25] To confirm the effect of the guanidium linkage of the AgTgAgTgAgT on the thermal stability (T_m) of the duplexes, the T_m values for an unmodified DNA·DNA duplex was also determined. As expected, AgTgAgTgAgT binds to DNA with much higher affinity (T_m = 56 °C) than complementary DNA (extrapolated T_ms to 5 °C, data not shown). Although the AgTgAgTgAgT·DNA duplex (Fig. 3) exhibited broad melting transition states, this anomaly is similar to DNA·RNG melting curves.[25,26] This can be explained by the electrostatic interaction between the positive charge of DNG and the negative charge of DNA, which provides an additional bonding, in spite of breaking down the Watson–Crick base pairing.

To study the sequence specificity of the binding of DNG with complementary DNA, AgTgAgTgAgT was allowed to form duplexes with complementary DNA (AgTgAgTgAgT·AATATA) and single-mismatch (5′- or 3′-end) DNAs (AgTgAgTgAgT·TATATT). These



Scheme 1. Synthesis of AgTgAgTgAgT phosphoramidate.

Table 1
Oligonucleotides DNG/DNA chimera guanidinium linkages indicated as 'g'

Compound	DNG/DNA chimeras	Yields (%)	Calculated (M)[a]	Founded (*m*/*z*)[b]
4	5′-CGCACGCAgTgAgTgAgTGTGGCGC-3′	21	6207.53	6208.61
5	5′-**G**GCACGCAgTgAgTgAgTGTGGCGC-3′	18	6247.47	6248.53
6	5′-CGCACGCAgTgAgTgAgTGTGGCG**G**-3′	19	6247.47	6249.02
7	5′-**G**GCACGCAgTgAgTgAgTGTGGCG**G**-3′	17	6287.49	6288.76
8	5′-CGCACGCATATATGTGGCGC-3′		Control	

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

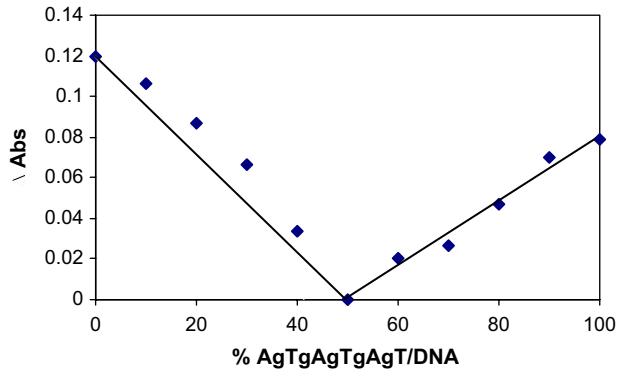


Figure 2. Job plot[27] mixing curves of the change of absorbance at 260 nm (ΔA_{260}) of aqueous solutions (pH 7.0, μ = 0.12 at 20 °C) with varied mole fractions of DNG and DNA ([AgTgAgTgAgT] + [DNA] = 2.0 μM). The inflection point indicates the stoichiometry to be a AgTgAgTgAgT/DNA duplex.

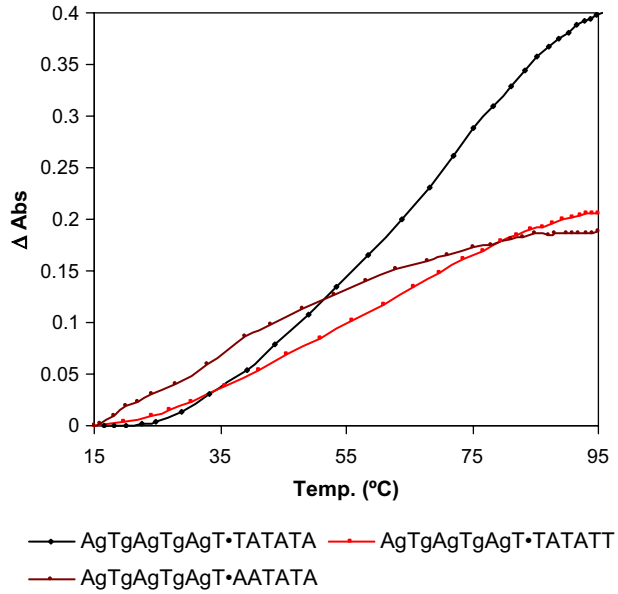


Figure 3. Melting studies; thermal denaturation curves for AgTgAgTgAgT and DNA duplexes. Absorbance was measured at 260 nm; the concentration of each strand was 6 μM in 10 mM Na_2HPO_4, 100 mM NaCl, pH 7.1. (Guanidinium linkages were indicated as 'g'.)

single-mismatch duplexes exhibit large decreases in T_m in comparison to the fully complementary AgTgAgTgAgT·TATATA duplex (Fig. 3). Thus, AgTgAgTgAgT maintains base-pair specificity while dramatically increasing its affinity for DNA compared with the standard DNA·DNA complex.

The melting temperatures and thermodynamic parameters of DNG/DNA chimeras (**4**–**8**) are provided in Figure 4 and Table 2.

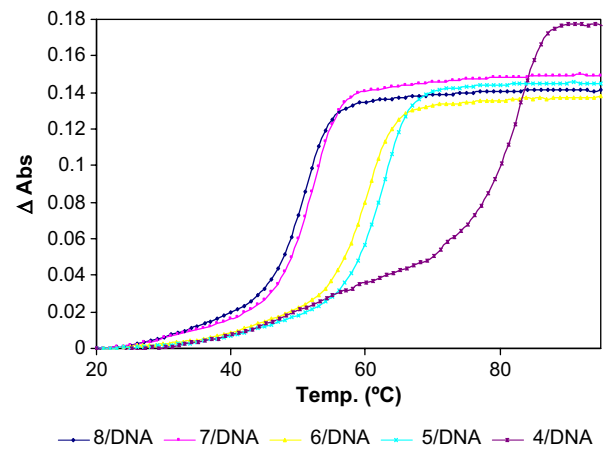


Figure 4. Melting studies; thermal denaturation curves for DNG/DNA chimeras. Absorbance was measured at 260 nm; the concentration of each strand was 6 μM in 10 mM Na_2HPO_4, 100 mM NaCl, pH 7.1. (Guanidinium linkages were indicated as 'g'.)

All experimental conditions employed in melting studies of DNG/DNA chimeras **4**–**8** were exactly the same as those of melting studies of AgTgAgTgAgT·DNA duplexes. As expected, Chimeras **4** with five charged guanidium linkages in 20-mer DNA binds to complementary DNA with much higher affinity (T_m = 75.0 °C) than DNA (T_m = 48.3 °C) (Table 2). As shown in Table 2, chimeras **5**, **6**, and **7** have the same number of guanidium linkages but have single- or double-mismatched base pairs in different positions. Duplexes **10** and **11**, which contain a single-mismatched base pair at 5′- or 3′-end, show 15.7, 18.3 °C decreases in T_m, respectively. The T_m value of duplex **12** with two-mismatched base pairs at 5′- and 3′-end has been lowered dramatically. However, it is still slightly more stable than unmodified DNA duplex.

Thermodynamic calculations were performed in order to describe the duplex binding more quantitatively. Standard free energies (ΔG°) for duplex formation are presented in Eqs. 1–4.

$$\text{DNA(control)} + \text{DNA} \xrightleftharpoons{\Delta G^\circ = -53.9\ \text{kJ/mol}} \text{DNA} \cdot \text{DNA} \quad (1)$$

$$\text{DNG/DNAchimera, } \mathbf{12} + \text{DNA} \xrightleftharpoons{\Delta G^\circ = -59.3\ \text{kJ/mol}} \mathbf{12} \cdot \text{DNA} \quad (2)$$

$$\text{DNG/DNAchimera, } \mathbf{11} + \text{DNA} \xrightleftharpoons{\Delta G^\circ = -63.9\ \text{kJ/mol}} \mathbf{11} \cdot \text{DNA} \quad (3)$$

$$\text{DNG/DNAchimera, } \mathbf{9} + \text{DNA} \xrightleftharpoons{\Delta G^\circ = -77.6\ \text{kJ/mol}} \mathbf{9} \cdot \text{DNA} \quad (4)$$

The $\Delta\Delta G^\circ$ of −23.7 kJ/mol (ΔG° Eq. 4–ΔG° Eq. 1) and −18.3 kJ/mol (ΔG° Eq. 4–ΔG° Eq. 2) translates into an increase in binding of many orders of magnitude. Thus, the binding of DNG/DNA chimera **9** to its DNA template is $>10^{5.7}$ tighter than the binding of the comparable DNA duplex. The tremendous increase in the free energy of binding of DNG/DNA chimera **9** (Table 2) supports the powerful attraction between the positively charged DNG guanidium groups and the negatively charged DNA phosphates. This increase is attributed to

Table 2
Melting temperatures and thermodynamic parameters for helix–coil transitions of DNG/DNA chimeras with DNA

DNG/DNA chimera	Duplex[a]	100 mM NaCl	T_m[b] °C	$-\Delta G^{\circ}_{25}$[c] kJ/mol
4	**9**		75.0	77.6
5	**10**		59.3	67.6
6	**11**		56.7	63.9
7	**12**		49.6	59.4
8	Control		48.3	53.9

[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).[26]
[c] Thermodynamic parameters were calculated by the method of Gralla and Crothers.[27,28]

both the formation of hydrogen bonds[28b] and favorable electrostatic attraction between DNG and DNA backbones.

From the above discussion, the following conclusions can be drawn about AgTgAgTgAgT and DNG/DNA chimeras: (i) The hexameric DNG, AgTgAgTgAgT, binds to a DNA strand in a 1:1 ratio; (ii) due to electrostatic attraction, a AgTgAgTgAgT DNG in complex with DNA (T_m = 56 °C) shows much more stability than DNA·DNA duplexes (extrapolated T_ms of 5 °C); (iii) the AgTgAgTgAgT DNG is able to discriminate between complementary and noncomplementary base pairs; (iv) in melting studies, the binding of a 20-mer DNG/DNA chimera that includes AgTgAgTgAgT is more than $10^{5.7}$ stronger than the binding of 20-mer DNA and also shows sequence specificity. It has been strongly suggested that thermodynamically favorable DNG/DNA chimeras may serve as potent antisense candidates. In vitro studies with yeast cells using DNG AgTgAgTgAgT oligomers and DNG/DNA chimeras will follow in order to evaluate the efficacy of guanidinium linked antisense agents.

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17. *m/z* (FAB) 1659.84, Calcd for $C_{69}H_{103}N_{36}O_{14}$ $(M+H)^+$ 1661.01.
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19. To a mixture of oligonucleotides **2** (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 was done with ethyl acetate followed by dilution with water, brine, drying over anhydrous Na_2SO_4 and evaporating under reduced pressure. The residue was purified by RP-HPLC (Altech Macrosphere RP C8 column 0–40% acetonitrile in 3% aqueous acetic acid). *m/z* (FAB) 2160.10, Calcd for $C_{100}H_{140}N_{38}O_{16}P$ $(M+H)^+$ 2161.40.
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21. *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, namely, CPG support and base protected 5′-*O*-(4,4′-dimethoxytrityl)deoxyribonucleoside-3-[*O*-(diisopropylamino)-β-cyanoethylphosphoramidite] 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 of the modified phosphoramidite oligomer 9,12, a coupling efficiency of >95% was observed for this step.
22. *Stoichiometry of binding (Fig. 2)*: The stoichiometry of binding was determined by the method of continuous variations. Solutions ([DNG] + [DNA] = 2.0 μM) containing different molar ratios of DNG 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®).
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24. *Melting Studies (Figs. 3 and 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. Concentrations of oligonucleotides were determined spectrophotometrically from molar extinction coefficients. 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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