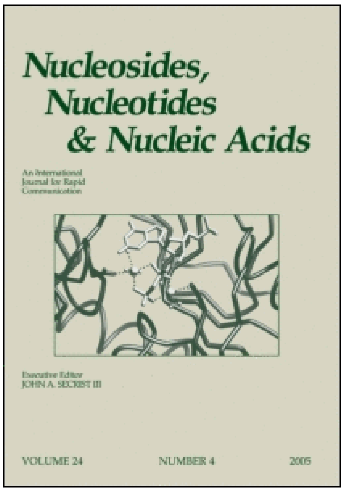

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Krishna N. Ganesh[a]; Kallanthottathil G. Rajeev[a]; Pradeep S. Pallan[a]; Vipul S. Rana[a]; Dinesh A. Barawkar[a]; Vaijayanti A. Kumar[a]
[a] Division of Organic Chemistry, National Chemical Laboratory, Pune, India

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MODULATION OF DNA TRIPLEX STABILITY THROUGH NUCLEOBASE MODIFICATIONS

Krishna N.Ganesh*, Kallanthottathil G.Rajeev, Pradeep S.Pallan, Vipul S Rana
Dinesh A.Barawkar and Vaijayanti A.Kumar

Division of Organic Chemistry, National Chemical Laboratory, Pune 411008, India

ABSTRACT

Spermine conjugation at N^4 of 5-Me-dC in oligonucleotides (*sp*-ODNs) reduces the net negative charge and these as HG strands form triplexes with foremost stability at neutral pH (7.3), in contrast to unmodified ODNs which form stable triplexes at pH 5.5. The stability of sp-ODN triplexes is shown to arise from improved association with duplex caused by electrostatic interaction of polycationic spermine sidechain with anionic phosphate backbone of DNA and N3 protonation is not a pre-requirement for triplexes constituted from *sp*-ODNs. The amplification of electrostatic component of interaction can be achieved by transformation of primary amino group of polyamines to corresponding guanidinium functions leading to improved binding and stabilization of DNA triplexes even at pH 7.0. %-Amino-dU ODNs are shown to be compatible as a central strand in formation of triplexes in which pyrimidine would be in the middle position of a triad.

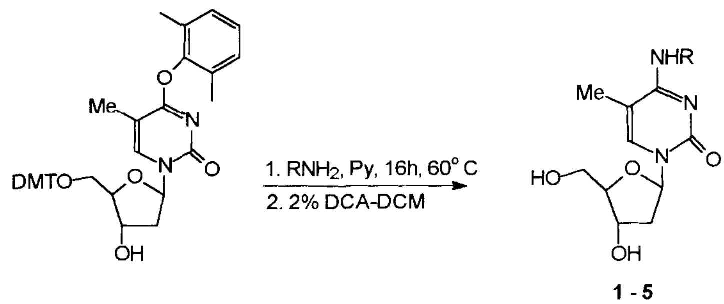
INTRODUCTION

The past several years have witnessed remarkable progress in the development of antisense oligonucleotides as pharmacological tools and as therapeutic agents.[1] The significant limitations with natural DNA sequences such as nuclease susceptibility and low permeability has resulted in synthesis of a variety of DNA structural analogues.[2] These comprise of chemical modifications at phosphate backbone, sugar residues and the heterocyclic nucleobase. The emphasis is on retaining affinity and specificity of oligonucleotides towards target DNA duplex (antigene) and RNA (antisense) while improving cellular stability and uptake. Serious drawbacks of antigene approach based on formation of triplex DNA include (i) the necessity of protonation of third strand C at N3 to recognize G of duplex GC base pair with the consequence that triplex formation is most favoured at non-physiological pH of 5.8 and (ii) requirement of a purine (A/G) in the central strand.[3] This has led to introduction of C5 substituents in dC to modulate the pK_a of N3, design of neutral mimics of protonated C and use of Ψ-/Ψ-iso pyrimidines in the central strand.[3b] Spermine is known to favour triple helix formation when present in millimolar concentrations and it was recently shown that spermine conjugation at 5'-end of oligonucleotides led to improved triple helix stability[4] at pH 6.5. In view of the positive attributes of 5-Me-dC and spermine in promoting triple helix formation, it was thought that a combination of both in the same residue as in **1** would have a constitutive effect on triplex formation at physiological pH and this rationale was indeed supported by experimental results.[5] This paper examines the possible chemical origin of this stability and amplification of the electrostatic stabilization at pH 7.0 through the use of guanidinium functions.

RESULTS AND DISCUSSION

Non-protonation of N3 in sp-ODN triplexes: pK_a measurements and UV spectral studies

The pK_a of N3 was considered to be one of the critical factors in influencing the stability of triplexes.[3] It is well known that substituents at C5 (Me, Br) would considerably affect pK_a of N3 and triplexes containing 5-Me-dC in HG strand form triplexes with better stability compared to triplexes with dC in third strand. We sought to examine the effect of N^4-substituents on N3 pK_a in nucleosides, oligonucleotides and the derived triplexes. 5'O-DMT-5-Me-dC containing various N^4-substituents such as propyl (**2**), butylamino (**3**), trioxyethylene (**4**) and spermine (**1**) were synthesised from O^4-dimethylphenyl-5'-dimethoxytritylthymidine (Scheme 1) by treatment with corresponding amines. The resulting N^4-substituted derivatives of 5'O-DMT-5-Me-dC were acid-deprotected to obtain the N^4-substituted nucleosides (**1-4**). The pK_a of N3 in these modified nucleosides were determined by pH-titration of their individual aqueous solutions with alkali (Table-1) and it is seen that N^4 substitution has a marked effect on N3 pK_a. Substitution with a simple alkyl chain (entry 2) does not affect the pK_a of N3 as compared to 5-Me-dC (entry 5) while the presence of even a simple amino group in the side chain lowers the pK_a by 0.5-0.6 units as observed for 5-Me-dC-(N^4-butylamine) (entry 3). Increasing the number of amino groups in the side chain as in 5-Me-dC-(N^4-spermine) does not have any further effect on pK_a of N3 (entry 1). The role of easily protonated N^4-side chain amino groups in lowering the pK_a of N3 was confirmed by the measured value for 5-Me-dC-(N^4-triethyleneoxy) (entry 4) devoid of sidechain amino group, which had a pK_a more like that of alkyl dC nucleosides. The lower



Scheme 1

TABLE 1: pK_a of N3 in N^4-substituted nucleosides

	R	pK_a
1	$(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$	3.7
2	$CH_2CH_2CH_3$	4.5
3	$CH_2CH_2CH_2CH_2NH_2$	3.8
4	$(CH_2)_2O(CH_2)_2O(CH_2)_2O(CH_2)_2OH$	4.0
5	H	4.4

N3 pK_a of nucleosides carrying amino groups in N^4 side chain may arise due to the fact that prior protonation of alkylamino group (with a higher pK_a) may disfavour further protonation at N3. In case of N^4-spermine analog, even the lowest pK_a value of spermine (8.03) is much higher than the N3 pK_a and hence does not interfere in pK_a measurements.

5-Me-dC-(N^4-spermine) was incorporated into oligonucleotides at specific sites via suitably protected 3'-O-phosphoroamidites to obtain *sp*-ODNs **10-14**. These formed stable triplexes with the complementary duplex **6:7** as confirmed from the biphasic dissociations in the UV- T_m curves.[5] The pK_a value of N3 in the oligonucleotides **8** and **14** in single strand and triplex form with duplex **6:7** were measured by acid-base UV-titration. The pK_a of N3 in dC of ODNs **8** and **14** was found to be enhanced in relation to that in monomer by 0.3 to 0.5 units respectively. A direct proof for protonation of N3 was sought from the known fact that dC shows characteristic near-UV spectral changes with appearance of a band at 288 nm upon protonation. As shown by this assay, significant protonation of N3 occurred for dC in oligonucleotides, both in single strand and in triplex form. Such a behaviour was not seen for 5-Me-dC-(N^4-spermine) oligonucleotides at pH 7.0, in single strand and triplex forms. The UV spectral band characteristic of N3 protonation in dC (288 nm) was enhanced at acidic pH

(<6.0) and shifted to 298 nm in dC-(N^4-spermine) and 294 nm in *sp*-ODN. These results clearly pointed to non-protonation of N3 in *sp*-ODN triplexes at physiological pH.

Electrostatic effects and hysteresis

The polycationic spermine sidechain can interact with anionic phosphate backbone leading to a diminished net charge for *sp*-ODNs as evident from observed retardation in their electrophoretic migration on gel.[7] This behaviour, as expected from a counter-ion condensation effect, is similar to that seen earlier for zwitterionic DNA by Switzer *et al* [8] and arises due to electrostatic interactions. While the heating and cooling curves of *sp*-ODN triplexes were nearly superimposable, that of unmodified control triplex (**8*7:6**) was irreversible in triplex ⇔ duplex transition, with the cooling curve exhibiting incomplete association of triplex even at 5^0C.[5b] In triplexes, the association of third strand to duplex during cooling is slower than the thermal dissociation leading to hysteresis effect. The contrasting behaviour of *sp*-ODN triplexes which show lack of hysteresis suggests that the third strand association is relatively enhanced compared to that of unmodified triplex. This enhancement in *sp*-ODN association is clearly due to favoured electrostatic interaction of polycationic spermine of third strand with the negative potential of the DNA duplex. The increased stability of *sp*-ODN triplexes is also reflected in their capacity for mismatch tolerence: *sp*-ODN **12** formed triplex with duplexes containing the complementary doublets CG and AT, but not TA (Table-2). Any factors that strengthen electrostatic interactions should therefore contribute to triplex stability in a major way.

DNA triplex stabilization by bisguanidinium anlogues of polyamines

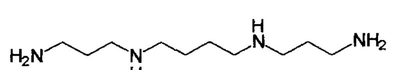
Guanidinium group is most commonly used by proteins and enzymes to recognize and bind anions through ion-pairing and hydrogen bonding which is favoured by the high basicity of guanidinium groups ($pK_a \sim 13.5$). We reasoned that spermine and spermidine analogues containing terminal guanidino groups as in SPMG **17** and SPDG **18** may possess biochemical and biophysical attributes well amplified due to a near total protonation at pH 7.0 in comparison to a lower degree of protonation of spermine/spermidine at identical pH. The bisguanidines **17** and **18** were synthesized by individually treating spermine (**15**) and spermidine (**16**) with the guanylating reagent N,N'-(bisbenzyloxycarbonyl)-S-methyl isothiourea[9] to obtain the corresponding protected N,N'-bisguanidinium derivatives, followed by removal of the benzyloxycarbonyl group by catalytic transfer hydrogenation. The biophysical effect of guanidinium groups in **17** and **18** was examined by UV- T_m binding

TABLE 2: UV-T_m of triplexes in the absence (-) and presence (+) of $MgCl_2$

6	3'	C	G	G	T	T	C	T_6	Y	T_6	C	T	G	C	G	(-)	(+)
7	5'	G	C	C	A	A	G	A_6	Z	A_6	G	A	C	G	C		
8				5'	T	T	C	T_6	C	T_6	C	T				-	28
9							*C*		*C*		*C*					-	46
10							X		C		C					40	47
11							C		C		X					40	46
12							C		X		C					33	41
13							X		C		X					33	40
14							X		X		X					25	31
a							C		X		C					29	36
b							C		X		C					27	40
c							C		X		C					nd	nd

* C = dC; *C* = 5-Me-dC; X = 5-Me-dC-(N^4-spermine); Y:Z, (a) C:G, (b) A:T (c) T:A

studies on the 24-mer DNA duplex (**6**:**7**) in TRIS buffer at pH 7.0. The bisguanidinium derivatives **17** and **18** enhanced the T_m of the 24 base pair duplex by 7-8°C over control (without any polyamine) and by 4°C over that with parent polyamines. No discrimination occured in DNA binding among the two polyamines or their guanidinium analogues at pH 7.0.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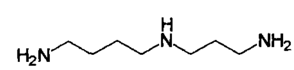
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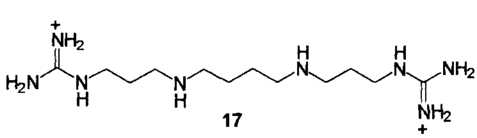
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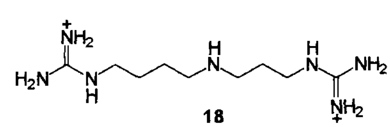
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In comparison to duplex results, profound effects were seen for triplex stability. While in the absence of spermine or spermidine no triplex formation was seen at pH 7.0, in presence of polyamines, triplexes were observed with the following stability order **16** < **18** < **15** < **17**. Thus the transformation of terminal amino groups of spermine to guanidino function (**17**) resulted in a significant stabilization of triplex **8*7:6** by 12.5°C over spermine (**15**) at pH 7.0. The corresponding transformation of primary amino functions of spermidine enhanced the T_m of triplex by only 5.5°C. Since triplex transformation in polypyrimidine motif is favoured at acidic pH, the T_m experiments were also carried out at pH 5.5. Under these

TABLE 3: Triplex DNA UV-T_m in presence of polyamine analogues*

pH	Control	SPM, 15	SPMD, 16	SPMG, 17	SPDG, 18
7.0	nd	36.0	26.0	48.5	31.5
5.5	34.5	46.0	33.5	51.5	45.0

* *Triplex* **8*7:6**

conditions, triplex formation was observed even in the absence of any polyamines and in their presence, the bisguanidinium **17** exhibited greatest triplex stability (ΔT_m, 17.0). The effect of acidic pH in stabilization of triplex over neutral pH is relatively large for spermine **15** (ΔT_m, 10°C) as compared to the corresponding bisguanidine **17** (ΔT_m, 3°C). Such pH induced stabilization was almost negligible on duplex. These results strongly suggest modulation of electrostatic interactions as a potential strategy for molecular engineering of DNA triplexes stable at physiological conditions, as needed in therapeutic applications.

DNA triplex formation with 5-amino-dU in central strand

The second requirement of purines at the central position of a triplex triad may be overcome by use of purine mimics designed to form hydrogen bonds from both WC and HG sides. 5-Amino-dU ($U^{\#}$) is such an engineered pyrimidine and the WC base pair $U^{\#}$:A can bind to purine A or G in third strand of a triplex via 5-amino group.[10] This modified nucleoside was incorporated at specific sites into the central strand of a DNA triplex and the stability of triplexes were monitored by temperature dependent UV absorbance change. The data indicated an interesting discrimination in molecular recognition of $U^{\#}$ located in central strand by third strand A and G. Formation of stable DNA triplexes were noticed only when A containing third strand was parallel to central strand **(19*21:22)** and the G third strand was antiparallel **(20*21:22)**. Both triplexes exhibited a higher T_m compared to corresponding control T analogues. The pyrimidine motif triplexes containing the triads A*$U^{\#}$:A and A*T:A were formed only at pH 5.8 and not detected at pH 7.0. However, the antiparallel purine motif triplex **(20*21:22)** without any C in third strand was observed at both pH, the stability being slightly more at pH 5.8. The results can be rationalised by hydrogen bonding scheme shown in Figure 1. Modified ψ-bases have recently been used as central bases of triple triads[11] and this allows formation of triplexes at single strand target sites of unrestricted sequence employing two oligonucleotide probes, one of which contains modified pyrimidines. Our present report on use of 5-amino-dU adds a new repertoire to nucleic acid recognition.

TABLE 4: UV-T_m of 5-Amino-dU Triplexes*

Entry	Triad X*Y:Z	Triplex	Tm (°C) pH 5.8	pH 7.0
1	A*U#:A	**19*21:22**	28	nd
2	G*U#:A	**20*21:22**	35	37

* Buffer: 100mM Sodium cacodylate, 20mM $MgCl_2$, 1M NaCl

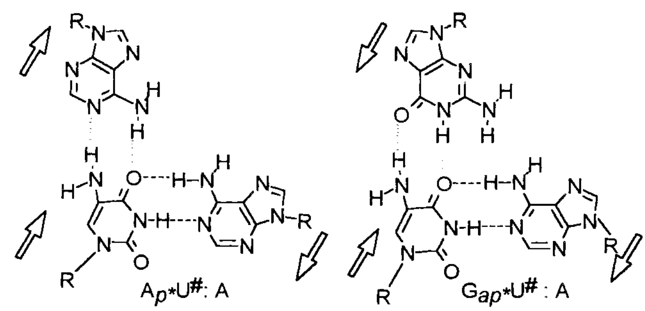

Figure 1

19			5'	T	T	C	T	T	T	T	X_p	T	T	C	T	T	C	T		
20			3'	A	A	G	A	A	A	A	X_{ap}	A	A	G	A	A	G	A		
21	5'	C	G	A	A	G	A	A	A	A	**Y**	A	A	G	A	A	G	A	C	C
22	3'	G	C	T	T	C	T	T	T	T	**Z**	T	T	C	T	T	C	T	G	G

CONCLUSIONS

In this article, it is demonstrated that the two limitations in applications of DNA triplex formation as a therapeutic strategy can be conceptually addressed by (i) conjugation of a polyamine to nucleobase to yield triplexes at pH 7.0 and (ii) use of a purine mimic such as 5-amino-dU in central strand. Amplification of electrostatic interactions is possible by use of polyguanidiniums which are mostly in cationic form at pH 7.0 instead of polyamines and this may improve cell permeablity[12] of therapeutic oligonucleotides. Efforts are underway to fully establish these principles and exploit the same for practical advantages.

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