Pure & Appl. Chem., Vol. 70, No. 2, pp. 289–292, 1998.
Printed in Great Britain.

Enhancing DNA triplex stability via nucleobase modifications

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

Division of Organic Chemistry, National Chemical Laboratory, Pune 411 008, INDIA

Abstract: This paper demonstrates that covalent conjugation of polyaminic ligands to nucleobase C in the third strand enhances DNA triplex stability at physiological pH and that a modified pyrimidine 5-NH_2-dU can be accommodated in the central position of a triplex triad. The chemical and biophysical basis for the observed stabilities are discussed and the results have significance in designing oligonucleotides for therapeutic applications.

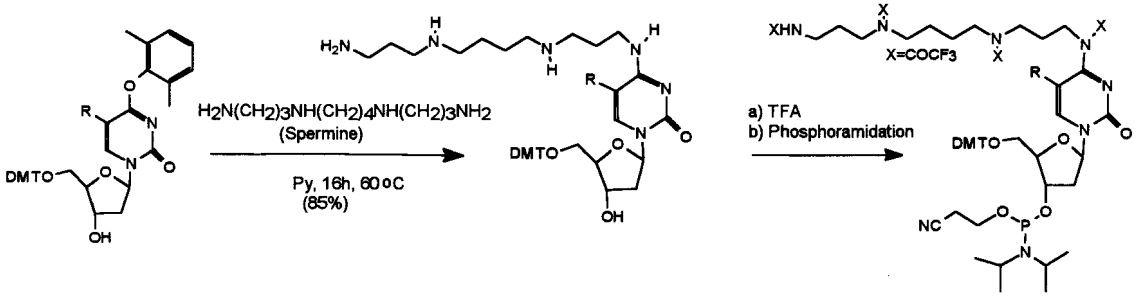
INTRODUCTION

Oligonucleotide-based drugs can theoretically be designed to (i) inhibit transcription by interaction with DNA duplex by triple strand binding[1] (antigene) or (ii) arrest translation by binding to m RNA target[2] (antisense). The success of this therapeutic approach requires that the potential oligonucleotide drug be stable to cellular nucleases and lipophilic enough to cross the cell membrane to enter cytoplasm. This has resulted in a significant number of chemical modifications of oligonucleotide structure at heterocyclic base, sugar and backbone functions. Such modifications must not only affect the affinity and specificity of binding to target DNA/RNA but also possess acceptable pharmacokinetic and toxicological properties to satisfy the demands of medicinal chemistry.[3]

The formation of DNA triple helices through binding of third strand in the major groove of DNA double helix via parallel Y* R:Y or antiparallel R* R:Y motifs has a direct potential for nucleic acid based therapeutics. This approach has limitations with natural nucleobases since optimal triplex stability is seen only at acidic pH and a purine base is necessary in the central position of triplex triad. We have employed the modified nucleobase 5-Me- dC-(N^4-spermine) to avoid the acidic pH requirement for triplex formations.[4] Apart from inducing triplex formation at physiological pH, the conjugation of polyamines to DNA may also facilitate cellular uptake of DNA through polyamine receptors present on the cell surfaces.[5] In another application, the modified pyrimidine base 5-amino-dU is shown to mimic a purine in the central strand of the triple helix.[6]

CHEMICAL SYNTHESIS OF 5-Me-dC-(N^4-SPERMINE) OLIGONUCLEOTIDES

For site-specific conjugation of oligonucleotides, the convertible nucleoside approach[7] was used by which the O^4-dimethylphenyl-5'-O-dimethoxytrityl thymidine 1 was converted to 5-Me-dC(N^4-spermine) derivative 2 by treatment with the polyamine spermine. The amino groups on spermine residue were protected as trifluoro



1 2 3

acetyl derivative and then subjected to phosphoramidation. The resultant 3'-O-phosphoroamidite 3 was used for incorporation into oligonucleotides at desired position by automated solid phase DNA synthesis. The oligonucleotides were deprotected under standard conditions to obtain *sp*-ODNs whose purity was established by RP HPLC and the retention of spermine conjugation was indicated by FABMS. It was observed that the presence of polycationic conjugated spermine resulted in retardation of bands in PAGE, the magnitude of retardation being directly related to the number of spermine substitutions per ODN molecule (Fig. 1). These observations indicated the zwitterionic nature of *sp*-ODNs in agreement with that observed in related molecules[8] and completely established the chemical identity of *sp*-ODNs.

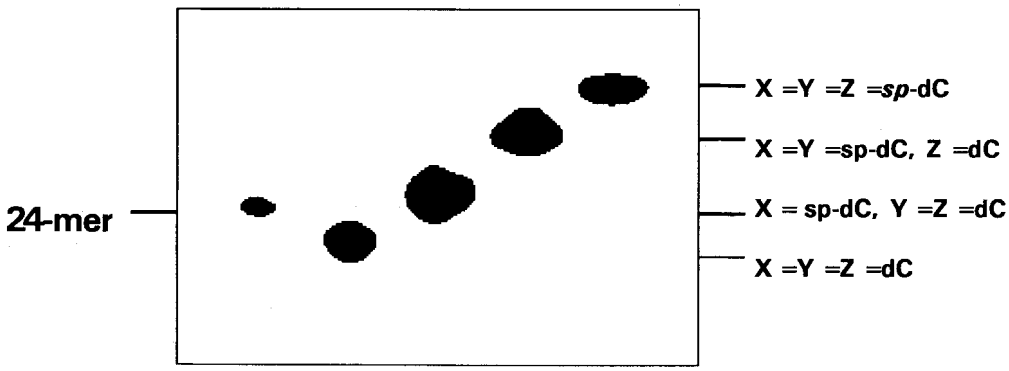


Fig. 1 Gel Retardation of *sp*-ODNs

DUPLEX AND TRIPLEX FORMATION BY *sp*-ODNs

The *sp*-ODNs were individually hybridized with complementary DNA strand and Tm of resulting duplexes were determined by temperature dependent UV absorbance measurements. *sp*-ODN conjugates formed duplexes with lower Tm (3-7°C) compared to the unmodified duplexes. This destabilization may result from a steric or electronic perturbation of WC base pairing induced by N^4 alkyl substitution. It is known that rotation of C4-N^4 bond in such nucleoside derivatives results in N^4 alkyl group adopting *syn* or *anti* alignment in relation to N3 of dC, with a preference for *syn*.[9] This may result in steric interference and lowering of duplex stability. Thus, the presence of protonated amino groups in spermine tether does not seem to provide any extra stability to the duplex in contrast to the well known stability imparted by external addition of spermine.

When the spermine chain is located at N^2 of G in the minor groove, a different situation exists leading to duplex stabilization.[10]

To examine the structural effects of *sp*-ODNs for antigene activity, triplexes bearing spermine on a third strand were generated by hybridization of *sp*-ODNs with a 24-mer designed duplex. It was observed that the *sp*-ODNs derived triplexes were much more stable compared to the control unmodified triplexes (Table 1). Among the different *sp*-ODNs, those containing spermine conjugation towards 5'/3'-ends gave better thermal stability compared to those with modification in centre. Significantly, triplex formation was observed even at physiological pH (7.0) and absence of $MgCl_2$. No triplexes were formed by unmodified ODN without addition of $MgCl_2$ under these conditions. The pH dependent triplex stability is critical for in vivo applications and such measurements indicated that while *sp*-ODN triplexes were most stable at pH 7.0, triplexes from dC and 5-Me-dC showed a similar stability only at acidic pH (5.5). This result has a direct bearing on practical utility of *sp*-ODNs.

The stringency of triplex formation by *sp*-ODNs were studied by their ability to hybridize with duplexes containing all four WC base pairs at positions complementary to 5-Me-dC(N^4-spermine) (X) location in third strand. Apart from X* G:C, other triplexes contain mismatched HG base pairing and the triplex stability order was X* G:C ~ X* A:T > X* C:G while no triplex were seen for X* T:A (Table 1). The hysterisis curve of *sp*-ODN triplex indicated a better association with complementary duplex as compared to unmodified ODN, indicating the positive role of spermine in promoting electrostatic interactions with anionic phosphates. UV difference spectral studies designed to investigate the protonation status of N3 suggested that unlike in normal

TABLE 1: UV-Tm of triplexes in the absence (-) and presence (+) of $MgCl_2$*

3' C G G T T C T T T T T T Y T T T T T T C T G C G	(-)	(+)
5' G C C A A G A A A A A A Z A A A A A A G A C G C		
5' T T C T T T T T T C T T T T T T C T	-	28
C *C*	-	46
X C C	40	47
C C X	40	46
C X C	33	41
X C X	33	40
X X X	25	31
a C X C	32	41
b C X C	29	36
c C X C	27	40
d C X C	nd	nd

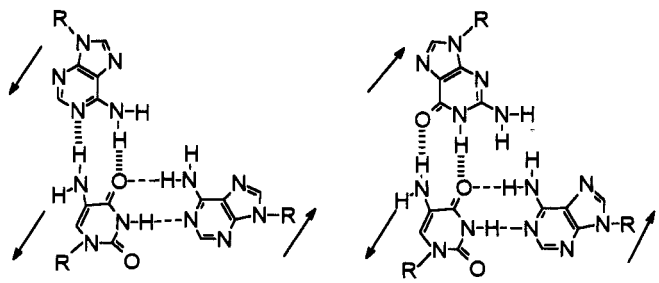
* C = dC; *C* = 5-Me-dC; X = 5-Me-dC-(N^4-spermine); Y:Z, (a) C:G, (b) G:C (c) T:A (d) A:T

triplexes requiring protonated C in third strand, no protonation of N3 occurs in 5-Me-dC(N^4-spermine), leading to absence of a single HG hydrogen bond in *sp*-ODN triplexes. Thus, the interstrand interaction of appended polycationic spermine more than compensates the loss in stability arising from absence of a HG hydrogen bond involving protonated N3.

The simple and site-specific spermine conjugation as described here permits introduction of polycations at internal sites in DNA and results in (i) imparting significant zwitterionic character to DNA (ii) formation of triplexes at pH 7.0 and (iii) increased stability of triplexes containing *sp*-ODNs as third strand. A lower net negative charge in *sp*-ODNs may assist cellular uptake either directly or through polyamine receptors present on cell surfaces.[5]

DNA TRIPLEX FORMATION WITH 5-AMINO- dU IN CENTRAL STRAND

The 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.[1] 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.[6] The 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.[6] The UV-Tm data indicated an interesting discrimination in molecular recognition of $U^{\#}$ in central strand by purines A and G. *$U^{\#}$ of WC base pair recognizes third strand A only in the parallel motif and G recognition occurs in the antiparallel motif.* The hydration pattern in major groove of DNA duplex is an important determinant for stability of antiparallel purine motif.[11] The replacement of hydrophobic 5-Me group of T by hydrophilic 5-amino function in major groove will have vital consequences in altering this hydration network to offer molecular discrimination of A and G. This finding adds a new repertoire to nucleic acid recognition which may be important in application of modified bases in central strand for dual recognition of single stranded target by two ODN probes.



$A_p * U^{\#}: A$ $G_{ap} * U^{\#} : A$

REFERENCES

1. (a) N. G. Thuong and C. Helene. *Angew. Chem. Int. Ed. Engl.* **32**, 666 (1993). (b) M. Cooney, G. Czernuszewicz, E. H. Postel, S. J. Flint and M. E. Hogan. *Science*, **241**, 456 (1988). (c) J. F. Milligan, M. D. Matteucci and J. C. Martin, *J.Med.Chem.* **36**, 1923 (1993) (d) L. J. Mahler, B. Wold, P. B. Dervan, *Science*, **245**, 725 (1989) (e) K N. Ganesh, V. A. Kumar and D. A. Barawkar, In *Perspectives in Supramolecular Chem.* (Vol.3). (A. D. Hamilton, ed), John Wiley & Sons Ltd. (1996) in press.
2. S. T. Crooke and B. Lebleu (Eds) *Antisense Research and Applications* CRC, Boca Raton, Ann Arbor and London Tokyo (1993).
3. S.T. Crooke and C. F. Bennett. *Annu.Rev.Pharmacol.Toxicol.* **36**, 107 (1996).
4. (a) D. A. Barawkar, V. A. Kumar and K. N. Ganesh. *Biochem.Biophys.Res.Comm.* **205**, 1665 (1994). (b) D. A. Barawkar, K. G. Rajeev, V. A. Kumar and K. N. Ganesh. *Nucleic Acids Res.* **24**, 1229 (1996).
5. R. J. Bergeron, W. R. Weimar, Q. Wu, J. K. Austin Jr. and S. McManis, *J. Med. Chem.* **38**, 425 (1995).
6. V. S. Rana, D. A. Barawkar and K. N. Ganesh, *J. Org. Chem.* **61**, 3578 (1996).
7. T. P. Prakash, D. A. Barawkar, V. A. Kumar and K. N. Ganesh, *BioMed. Chem. Lett.* **4**, 1733 (1994).
8. (a) H. Hashimoto, M. G. Nelson and C. Switzer, *J. Org. Chem.* **58**, 4194 (1993), (b) H. Hashimoto, M. G. Nelson and C. Switzer, *J. Am. Chem. Soc.* **115**, 7128 (1993).
9. J. D. Engel and P. H. von Hippel. *Biochemistry*, **13**, 4143 (1974).
10. J. -P. Behr and N. Schmid, *Tetrahedron. Lett.* **36**, 1447 (1995).
11. I. Radhakrishnan and D. J. Patel. *Biochemistry*, **33**, 11405 (1994).
12. KNG acknowledges Homi Bhabha Fellowships Council, Bombay and Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore for financial support.