Chemical regulation of DNA triplex properties: Nucleobase and backbone modified novel DNA analogues as structural probes*

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During the past decade, a new approach using DNA analogues as therapeutic agents is emerging in medicinal chemistry. This is based on regulating expression of genes of disease-related proteins/enzymes by blocking their transcription (*antigene*) or translation (*antisense*). It is effected through sequence-specific binding of complementary oligonucleotides to either DNA duplex via triplex formation to inhibit production of mRNA or interfere in the translation of the latter to proteins. Since oligonucleotides do not enter cells easily and are amenable to destruction by cellular nucleases, a variety of chemically-modified analogues of oligonucleotides are being designed, synthesized and evaluated for development as therapeutic agents. This article summarizes recent developments in this futuristic area with a focus on results from the author's laboratory.

EVER since the discovery of the double helical structure of 2'-deoxyribonucleic acid (DNA)[1] by Watson and Crick in 1953, the genetic chemical DNA has held centre stage in biochemical research. The development of automated chemical synthesis of sequence-defined oligonucleotides[2] not only accelerated genetic engineering but also has led to other innumerable applications such as structural investigation of DNA and ribonucleic acid (RNA)[3] and their complexes with drugs[4], proteins[5] by NMR spectroscopy[6] and X-ray crystallography[7], polymerase chain reaction (PCR)[8], drug discovery through combinatorial chemistry[9] and systematic evolution of ligands by exponential enrichment (SELEX)[10] and recently in therapeutics[11] and diagnostics[12]. Traditional medicinal chemistry involves development of drugs targetted against functional proteins to elicit a therapeutic response. The rational design of such molecules requires a lot of structural information about the target protein and structure–activity relationships with suitable analogues[13]. The recently-emerging nucleic acid based therapeutics (Figure 1) addresses regulation of gene expression *in vivo* by direct inhibition of the gene or mRNA through short complementary oligonucleotides[11]. Since the best way to recognize and inhibit the functioning of DNA or mRNA is through a complementary base sequence, this strategy envisages development of synthetic analogues of short oligonucleotides as new medicinal agents. The *antigene* strategy exploits the possibility of binding exogeneous nucleic acid analogues to duplex DNA of the gene in order to hamper mRNA synthesis on the DNA template while the *antisense* approach is directed against the functioning of mRNA template for the synthesis of proteins. Since a continuous gene expression keeps supplying new mRNA molecules, the antigene strategy to block the genes may be more efficient than the antisense approach. For any of these to be effective, the oligonucleotide analogues must conform to all requirements of a functional drug such as cell permeability, stability in the cells and bioavailability at the desired target (Figure 2).

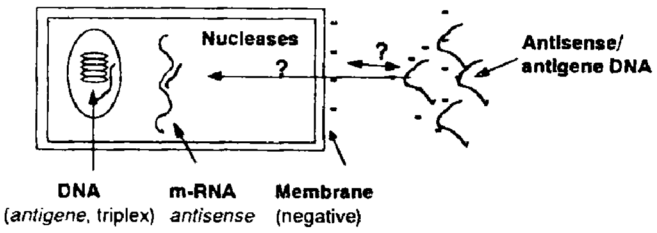

Figure 1. Principles of antigene and antisense therapeutics.

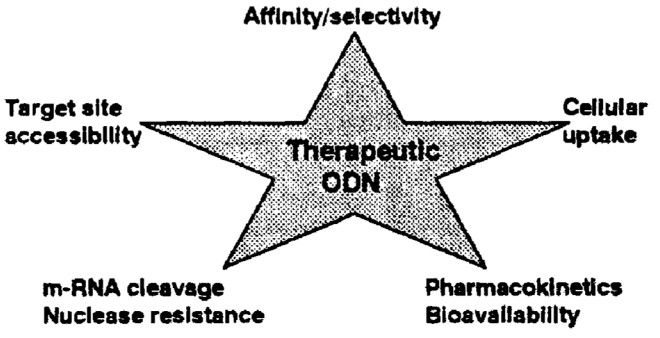

Figure 2. Technical hurdles in development of therapeutic oligonucleotides.

*Dedicated to Professor G. B. V. Subramanian, Delhi University, on his 65th birthday.

Table 1. Some diseases amenable for treatment by DNA therapeutics*

Disease	Cause
Cancer	Uncontrolled cell growth from mutational activation and expression of oncogenes
Viral infections	Replication of virus in host cells, e.g. HIV, HSC, Influenza
Endrocrinological	Abnormal levels of – renin, angiotensinase or vasopressin precursor (high blood pressure) – transforming growth factor (kidney failure) – growth hormone (acromegaly) – gastrin (ulcers)
Neurological	– lesins in β-amyloid gene (Alzheimer's disease) – overproduction of monoamine oxidase (mental illness)
Autoimmune	– inadvertant production of antibodies against normal tissues (degeneration of host tissue-arthritis, myastenia gravis), blocking β-cell Ig genes or T-cell receptor genes by antisense
Bacterial	Antibiotic resistant tuberculosis, mycoplasmas-blocking of 3′-terminus of 16 s RNA
Parasitic	Haem polymerase production (Malaria-blocking expression of haem polymerase), Sleeping sickness (Trypanosoma)

*These are only some examples to illustrate the potential of the approach. See ref 11.

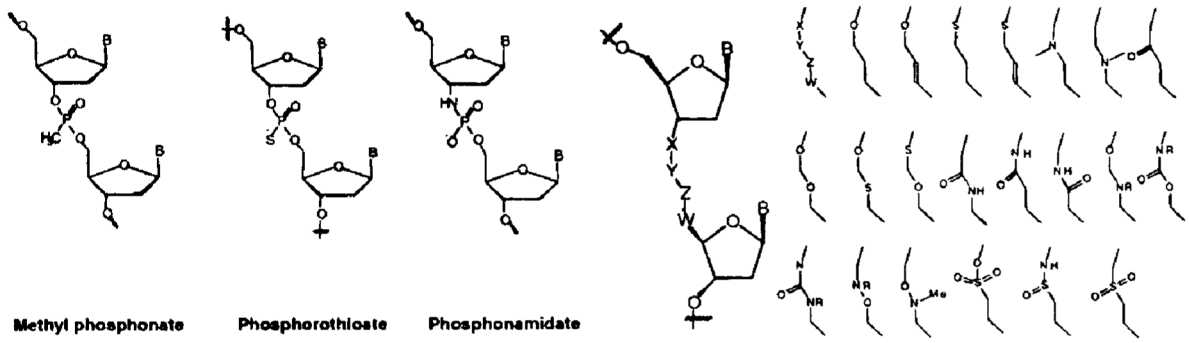

Figure 3. Chemical modifications of DNA.

Meeting the essential criteria of a drug offers formidable hurdles in case of oligonucleotides. Since these are negatively-charged polyions, their penetration into cellular membranes with anionic surfaces is not facile. Once they are within the cytoplasm, they become easy substrates for cellular nucleases and hence get degraded. The desired target for antigene action is a DNA duplex that is localized within the nucleus and always bound by proteins, which makes its molecular recognition via triplex formation a difficult process. These aspects rather than being limitations, have created new opportunities and challenges for medicinal chemists, to design DNA analogues that are nuclease-resistant and cell-permeable, without loss of target-binding specificity. The approach is based on modification of phosphate structure to phosphorothioates[14], methyl phosphonates[15] and phosphonamidates[16] or replacing the negatively-charged phosphate linkage with neutral groups such as amides, acetals, etc.[17] (Figure 3). Among these, the phosphorothioates have emerged as first generation antisense agents with success in a number of disease targets (Table 1). However, they have drawbacks in terms of non-specific binding to several proteins leading to toxicity[14] and being chiral at each P atom, n number of

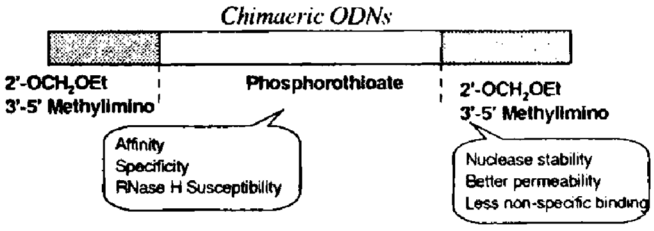

Figure 4. Second generation antisense oligonucleotides

phosphates in oligodeoxyribonucleotides (ODN) leads to a mixture of 2^n configurational isomers which is not desirable for development of drugs. The next generation of antisense agents will perhaps be based on a combination of several structural attributes (Figure 4), each addressed to overcome specific limitations. For example, resistance to 5′/3′-exonucleases may be imparted by introduction of 2′-O-alkylribo residues at the 5′ and 3′ ends whereas endonuclease resistance is conferred through phosphorothioate linkages in the core[18]. This together with recent development of elegant methods for stereoregulated synthesis of chirally pure (all R_p or S_p) phosphorothioates[19] and recruitment of appropriate cell delivery methods should pave the way for more efficient and selective antisense agents.

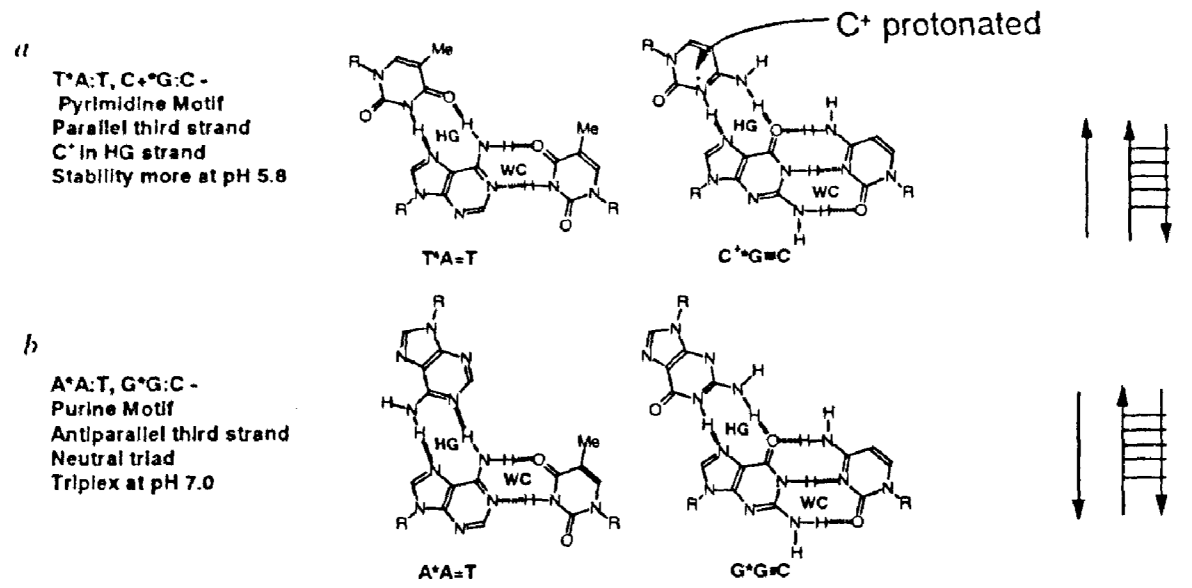

Figure 5. Hydrogen bonding scheme in DNA triple helix. *a*, Pyrimidine motif. *b*, Purine motif. In both, purine is always the central base.

Antigene therapeutics involve formation of triple-stranded DNA complexes arising from binding of an oligonucleotide to a target DNA duplex (Figure 5), thereby inhibiting the separation of duplex strands which is necessary for transcription of the gene[20]. During the past few years, triplex formation has been shown to inhibit transcription and subsequent protein synthesis in a number of experimental cell-free systems[20]. The triplex formation is based on recognition of Watson–Crick (WC) hydrogen bonded AT and GC base pairs in duplex by third strand T and C^+ respectively via Hoogsteen hydrogen bonds (Figures 5 *a* and *b*). In the arrangement, termed 'pyrimidine motif', the third strand remains parallel to the central purine strand. In the alternative 'purine motif', the third strand is comprised of a polypurine stretch in which A recognizes an AT base pair while G binds a GC base pair with the third strand association of duplex in an antiparallel fashion.

The prerequisites for this scheme of triplex formation are that in the pyrimidine motif, (i) the third strand C needs to be in protonated form which is possible only at non-physiological conditions (pH < 6.0) and (ii) purine is always necessary in the central position for forming H-bonds from either side and hence triplexes are confined to targetting polypurine stretches. Much of the work during the past decade in our laboratory[21] is aimed at chemical modifications that seek to overcome these two molecular limitations and the next section overviews our strategies and results.

Base modified oligonucleotides

The modification of nucleobases directly affects the recognition–complementation interactions of oligonucleotides and hence proper care has to be exercised in the design. We chose two base modifications that are not expected to affect the natural complementation specificity of the bases. These are linking a sperminyl moiety at N4 of dC (Figure 6) and replacement of 5-methyl group of T by an amino function to generate 5-amino-dU (Figure 7), a purine mimic. The application of these base modified oligonucleotides in stabilization of DNA triplexes is described here.

Spermine conjugated oligonucleotides

Spermine is known to favour triple helix formation when present in millimolar concentrations and this is due to the electrostatic neutralization of high negative potential in triplexes by the polycationic spermine[20]. We have improved this property of spermine by conversion of the terminal amino groups into guanidinium function[22] and by introduction of chirality through a conformational restraint. These spermine analogs showed better stabilization of triplexes. It was recently shown that spermine conjugation at the 5'-end of oligonucleotides led to improved helix stability at pH 6.5 (ref. 23). It is also known that 5-methyl-dC has a stabilizing effect on the triple helix when incorporated into the third strand of a DNA[23]. We envisaged that a combination of both in the same residue may have a constitutive effect on triplex formation at physiological pH and hence synthesized and incorporated *sp* (5-methyl-dC-N^4-spermine) (Figure 6 *a*) at different positions in oligonucleotides (*sp*-ODNs) designed to be a third strand in a triplex[24]. The formation of DNA triplex is conveniently monitored by temperature dependent UV (Figure 7 *a*) in which observance of a biphasic transition is indicative of the presence of

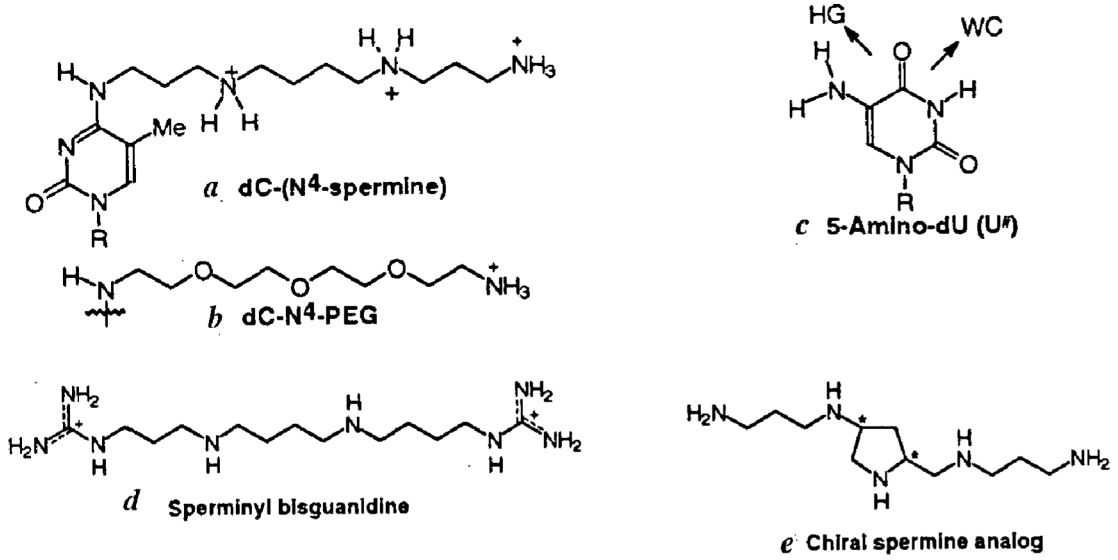

Figure 6. Structures of base modified nucleoside and spermine analogs.

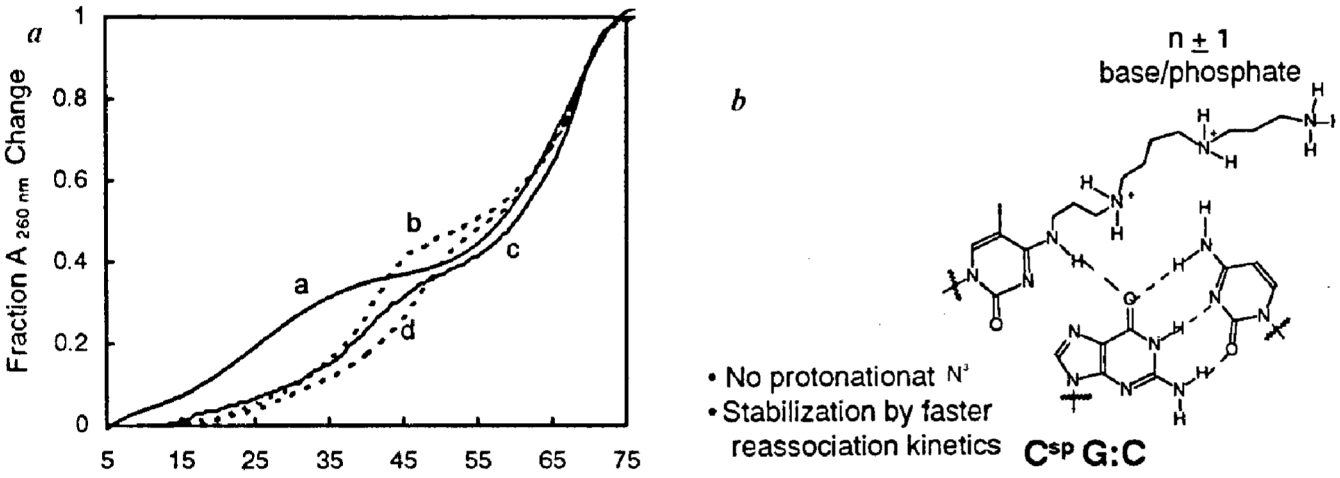

Figure 7. *a*, Typical biphasic UV melting profiles of triplexes. Examples are shown for *teg*-ODN triplexes (from ref. 26); *b*, Proposed schem for stabilization of triple helix by *sp*-ODNs.

Table 2. T_m of *sp*-ODN triplexes in the presence and absence of $MgCl_2$ at pH 7.0

DNA duplex																												
	C	G	G	T	T	C	T	T	T	T	T	T	C	T	T	T	T	T	T	C*	T	G	C	G		d		
d	G	C	C	A	A	G	A	A	A	A	A	A	G	A	A	A	A	A	A	G	A	C	G	C				
Third strand T_m																											$MgCl_2$	
																											–	+
d	T	T	C	T	T	T	T	T	T	T	C	T	T	T	T	T	T	T	C	T							–	28
			C*								C*								C*								–	46
			C#								C								C								40	47
			C								C								C#								40	46
			C								C#								C								33	41
			C*								C								C*								33	40
			C#								C#								C#								25	31

C* = 5-Me-dC; C# = 5-Me-N^4-(*sp*)-dC.

triplex with T_m corresponding to midpoint of low temperature transition[20]. The triplexes were constituted from hybridization of 18-mer *sp*-ODNs with a 24-mer duplex and stabilities at different pH values were compared to that of control derived from an unmodified third strand[25]. The results (Table 2) indicated that (i) triplexes with spermine conjugation towards 5′/3′ ends gave thermal stability better than those containing

modification in the centre, (ii) *sp*-ODNs formed DNA triplexes even in the absence of $MgCl_2$ which was necessary for observing triplexes from control and (iii) *sp*-ODN triplexes were most stable at pH 7.0 while unmodified 5-methyl-dC triplexes showed a similar stability only at acidic pH (5.5). The last property is very significant for antigene technology since simple spermine conjugation leads to triplex formation under physiological conditions, overcoming one of the limitations for observing triplexes.

The stringency of triplex formation by *sp*-ODNs was studied by their ability to hybridize with duplexes containing all four WC base pairs at positions complementary to the location of 5-methyl-dC-N^4-spermine (X). The stability order of triplexes containing 5-Me-dC-N^4-spermine was found to be X*G:C = X*A:T > X*C:G > X*T:A. The hysteresis curve of *sp*-ODN triplexes indicated a better reassociation with complementary duplexes as compared to unmodified triplexes and pH-dependent UV difference spectra suggested that N3 protonation is not a requirement for triplex formation by *sp*-ODN[25]. The origin of triplex stability of *sp*-ODNs can be attributed to favoured, additional electrostatic interactions with phosphates of the duplex and H-bonding with base pairs (Figure 7 *b*) *without any N3 protonation*. Thus, the interstrand interaction of conjugated spermine more than compensates for loss in stability due to absence of a single Hoogsteen hydrogen bond (N3-dC:N7-dA). This modification thus circumvents one of the limitations of triplex formation, which is the necessity of N3 protonation and hence stability of triplexes at only acidic pH.

To gain further insights into the factors that contribute to the enhancement of triplex stability and for engineering improved triplex systems, spermine appendage at C4 of 5-Me-dC was replaced with 1,11-diamino-3,6,9-trioxaundecane (Figure 6) to create 5-Me-dC-(N^4-tetraethylene-glycolmonoamine) (*teg*)-ODNs[26]. From the triplex forming abilities of these modified ODNs studied by hysteresis behaviour and the effect of salts on triplex stability, it was demonstrated that *teg*-ODNs stabilize triplexes through hydrophobic desolvation while *sp*-ODNs do so by charge effects. The results imply that factors other than base stacking and interstrand hydrogen bonds are significantly involved in modulation of triplexes by base modified ODNs such as *sp*- and *teg*-ODNs. The base modified ODNs were found to be good substrates for DNA polymerase when used as primers in chain elongation reaction and hence can be conveniently used in enzymatic preparations of desired DNA fragments for specific applications[26]. Since polyamines and polyoxyethylenes are well known to interact with membranes, these studies have significance in evolving newer strategies to improve cell permeability and in development of ligand conjugated ODN analogues as non-cytotoxic candidates for antisense/antigene therapeutics.

5-Amino-dU oligonucleotides

The critical 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 directions. 5-Amino-dU ($U^{\#}$) is such an engineered pyrimidine[27] and the WC base pair $U^{\#}$:A can bind to purine A or G in the third strand of a triplex via 5-amino group (Figure 8). This modified nucleoside was incorporated at specific sites into the central strand of a DNA triplex and the stability of triplexes monitored by temperature-dependent UV absorbance changes. The UV-T_m data indicated that stable triplexes are indeed formed with a pyrimidine in the central strand[28]. Further, an interesting discrimination in molecular recognition of $U^{\#}$ in the central position by purines A and G was noticed. $U^{\#}$ of WC base pair recognizes third strand A only in parallel motif and G recognition occurs in the antiparallel motif. Thus the triad A*$U^{\#}$:A is compatible with a pyrimidine motif while G*$U^{\#}$:A is compatible with a purine motif. The hydration pattern in the major groove of a DNA duplex is an important determinant for stability of the antiparallel purine motif[6]. The replacement of hydrophobic 5-methyl group of T by hydrophilic 5-amino function in the major groove will have vital consequences in altering this hydration network to offer molecular discrimination of A and G. The results add a new repertoire to nucleic acid recognition, with the necessity of a purine in the central position, no more a limitation for triplex formation in antigene therapeutics. This is also important in the application of modified bases in the central strand for dual recognition of single-stranded DNA target by two ODN probes[29].

Fluorescent oligonucleotides

Further, in this analogue, the 5-amino function could be useful to append intelligent ligands such as fluorescent groups, metallocomplexes, peptides, etc. to generate functionally useful oligonucleotides. We have linked the fluorophores dansyl[30] and 5/6-carboxyfluorescein[31] directly to the 5-amino group (Figure 9 *a*) to generate fluorescent ODNs that are useful in studying drug–DNA interactions, major groove polarity and in combination with PCR, for DNA diagnostics. The major and minor grooves of DNA have functional significance since they are sites of molecular recognition by DNA-binding agents such as proteins, drugs and metal complexes[3–5]. Small molecules such as drugs and some metal complexes bind DNA exclusively from the minor groove while a majority of DNA-specific proteins recognize DNA from the major groove side, sometimes with critical contacts in the minor groove. The fluorescence excitation and emission spectra of dansyl group in 5-amidodansyl-dU was found to be sensitive to the dielectric constant of the solvent medium[30]. Site-specific

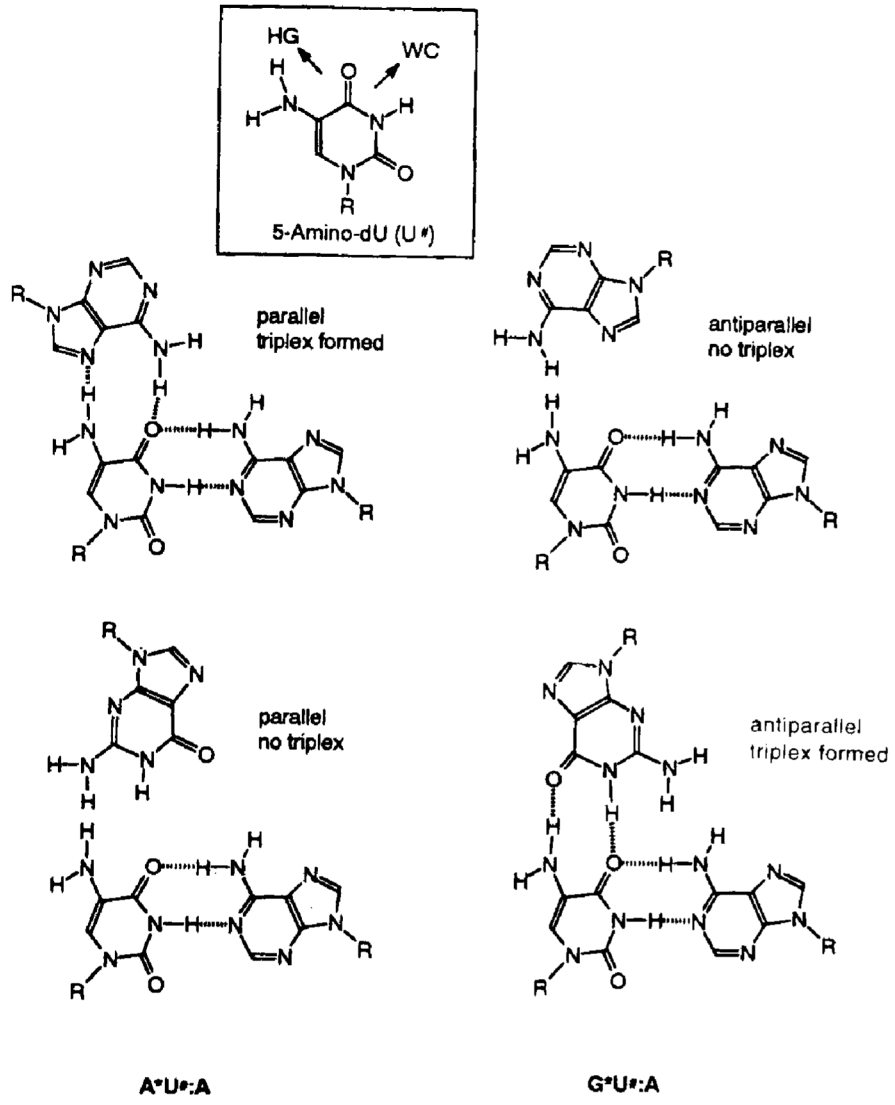

Figure 8. Triplex H-bonding scheme with 5-amino-dU in central strand.

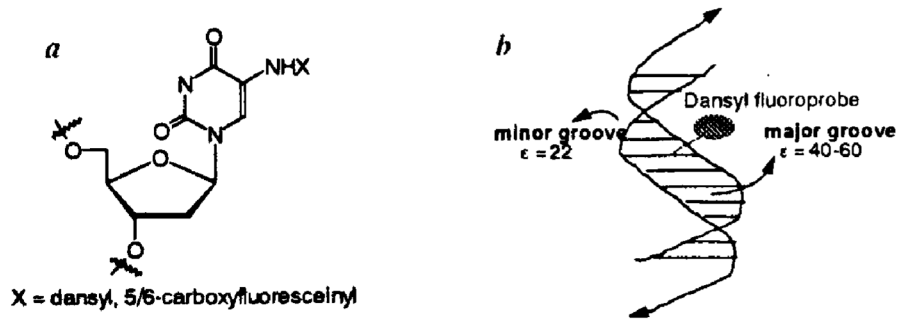

Figure 9. *a*, Fluorescent ODNs; *b*, Dielectric differential in DNA.

incorporation of 5-amididansyl-dU in ODNs leads to fluorescent DNA in which the reporter group resides in the major groove (Figure 9 *b*). The fluorescent observables from such a probe are used to estimate the dielectric constant of the major groove to be 55D, in comparison to that of the minor groove which is hydrophobic (24D). By using 5-amidodansyl-dU in different sequence contexts, we have recently found[12] that the

microenvironment in the major groove is dependent on the DNA sequence, with dielectric constant varying in the range 40–60. This has importance in understanding the hydration modes in DNA which is an important factor in controlling DNA structure, including salt-dependent transitions in various polymorphic structures[33]. An exclusive minor groove event such as DNA–netropsin association can be quantitatively monitored by fluorescence of the dansyl moiety located in the major groove[30]. It also suggests the existence of an information network (groove crosstalk) among the two grooves. The fluorescent DNA probes as reported here have potential applications in the study of DNA structure, DNA–drug and DNA–peptide interactions.

The fluorescein conjugated oligonucleotides give fluorescence in the visible region and are hence useful in the development of non-radioactive probes for DNA diagnostics. Since the sensitivity of the technique is poor compared to that offered by radioactive probes, we attempted at improving it by coupling it with an amplification technique such as polymerase chain reaction (PCR). By employing multilabelled fluorescent oligonucleotides (which is possible when the fluorophore is conjugated to a nucleobase such as 5-amino-dU) as primers in PCR, all amplified DNA is made fluorescent and visible to the naked eye[31]. The unreacted excess primers can easily be removed from high molecular weight product DNA by a simple filtration, even avoiding electrophoresis for detection. Since a wide variety of fluorophores with different emission characterics can be used, by a suitable choice of fluorescent primers, it is possible to apply this method for development of clinically useful and safer diagnosis of genetic diseases such as thalassaemia in humans and bovine herpes in cattles.

Backbone modified oligonucleotides

Chloramphenicol backbone containing oligonucleotides

The replacement of 2′-deoxyribose moiety of a nucleoside by acyclic chains has recently attracted attention[34] since they show resistance to enzymes. We thought of using chloramphenicol backbone (Figure 10 *a*) as acyclic synthons for substitution of sugar residue in nucleosides, as these are readily available and easy for synthetic manipulation. The chloramphenicol–nucleobase chimera was synthesized for site-specific incorporation into ODNs for triplex studies[35]. The UV–T_m studies indicated that these form triplexes with stability dependent on the number, position and stereochemistry of modification. These are perhaps the first examples of stable triple helices involving chiral acyclic moieties in the backbone of the third strand. The possibility of synthesis of different diastereomers gives an opportunity for stereochemical fine tuning of the backbone conformation for effective binding to ss/ds DNA via duplex/triplex formation. The substitution on the aryl ring may be further utilized as a handle for non-radioactive labelling or for conjugation of functional ligands in the backbone of oligonucleotides.

Peptide nucleic acids – Chiral, fluorescent and polyamine conjugates

Recently, a novel class of non-chiral, designed synthetic molecules – peptide nucleic acids (PNAs) (Figure 10 *b*), are emerging as potential antisense agents[36]. These have an ethylenediamine-glycine backbone to which the nucleobases are linked (at N) through an acetyl chain. PNA is chemically stable and in contrast to natural nucleic acids and peptides, PNA is expected to remain intact in living cells since it is not a substrate for natural hydrolytic enzymes and is not degraded in cell extracts. PNA has several properties that makes it promising for use as a gene targeting agent. PNA is capable of sequence-specific recognition of both DNA and RNA by WC base pairing and the hybrid complexes thus formed exhibit extraordinarily high thermal stabilities. It has been found that PNA invades DNA duplex in a sequence-specific manner, displacing the existing DNA complementary strand (Figure 10 *d*) and hence is ideal as a therapeutic agent. PNAs have a strong binding affinity to DNA, even higher than its complementary strand and hence may be ideal to target DNA within the nucleus and homopyrimidine PNAs form triplexes with PNA_2:DNA composition. However, PNAs have some drawbacks such as poor cell permeability and ambiguity in DNA recognition since these bind DNA in both parallel and antiparallel modes. This has restricted their widespread application in biology. We have conjugated PNAs with spermine at the C-terminus and such conjugates improved the solubility of PNA by 15 times and increased the binding affinity with DNA by 20 times due to a faster reassociation kinetics mediated by electrostatic interactions[37]. PNA analogues carrying the fluorescent 2-aminopurine have been synthesized and shown to be versatile for probing PNA–DNA hybridization kinetics[38]. By using conformationally constrained, chiral 4-aminoprolyl monomers[39] incorporated within the PNA chain (Figure 10 *c*), chirality was inducted into these molecules, resulting in better selectivity for DNA recognition. D-*trans* PNA binds DNA only in an antiparallel orientation with a B-form duplex, while L-*trans* PNA complexes DNA in a parallel mode and a A-form hybrid[39]. This clearly demonstrates that chiral induction into PNA enhances selective binding to DNA. Thus our chemical modifications provide solutions for addressing the two limitations of PNAs (solubility and ambiguous binding with cDNA) for biological applications.

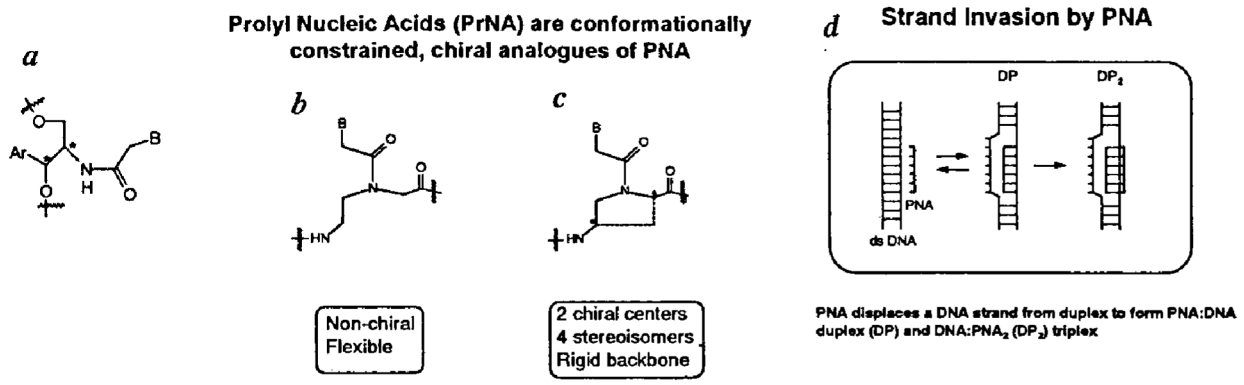

Figure 10. Backbone modified nucleic acids: (*a*) Chloramphenicol based, (*b*) Peptide nucleic acid (PNA), (*c*) Chiral prolyl nucleic acids and (*d*) Strand invasion by PNA.

Conclusions

Synthetic oligonucleotides containing rational modifications have emerged as new tools for practical applications in therapeutics and diagnostics apart from their utility in studying molecular recognition of DNA and its interaction with ligands. Chemical modification at nucleobases by tethering of 'intelligent' chemical functionalities has led to the construction of designer oligonucleotides for tailored applications. The duplexes and triplexes derived from these have potential advantages over natural DNA in terms of complementation, stability and membrane permeability. In particular, the linking of cationic spermine and polyethers lead to zwitterionic DNA having lower net negative charges and consequently a better membrane penetration. ODNs containing dC-(N^4-spermine) units were shown to form stable DNA triple helices at physiological pH (7.6). The design of DNA triplexes with such stability attributes at therapeautically relevant conditions is currently a most pursued topic. Further, since cell surfaces are endowed with polyamine receptors[40], spermine-ODNs may have a better potential for receptor-mediated cell uptake compared to standard ODNs. The pyrimidine 5-amino-dU is shown to function as a central base of a triad since it can form H-bonds from both sides of the heterocyclic ring. It recognizes A in the third strand when in a parallel orientation, but binds a third strand G only in an antiparallel orientation. Such molecular recognition-based selectivity in forming triplexes would be extremely interesting and useful. The results are valuable in evolving tactics for recognition of purine/pyrimidine mixed single strand ODNs in cells by extraneous addition of two ODNs, one containing 5-amino-dU forming the middle strand of a triplex. The lacuna of the high potential PNAs in terms of solubility and orientation ambiguity in DNA complementation have been addressed by conjugation of spermine and introduction of chirality into PNA, with encouraging results.

This article illustrates that a judicious combination of organic synthetic techniques with identification of structural problems in bio-organic and medicinal chemistry would not only bring newer structural insights but also has potential for development of products with practical benefits.

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ACKNOWLEDGEMENTS. I am highly indebted to all my students for their dedicated work. I thank my senior colleague Dr Vaijayanti Kumar who has made a significant contribution to our work in terms of ideas, experiments and critical discussion. I acknowledge the Department of Biotechnology and the Department of Science and Technology, New Delhi, for financial support and Jawaharlal Nehru Centre for Advanced Scientific Research for help.

Received 29 July 1998; revised accepted 15 October 1998