ABSTRACT

Chapter 1: Introduction

During the past decade, a new approach using DNA analogues as therapeutic agents has emerged in medicinal chemistry. This is based on regulating the gene expression 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 through interference in the translation of the latter to protein. Since continuous gene expression keeps supplying new mRNA molecules, the antigene strategy to block the genes may be more efficient than the antisense approach.

Triple helices can be classified into two structural motifs, (Figure 1) viz. (i) the 'pyrimidine motif' in which the third strand, primarily composed of pyrimidine bases, is parallel to central purine strand where AT base pair recognizes neutral T and GC base

![Figure 1: Pyrimidine motif (parallel)](image1)

![Figure 1: Purine motif (antiparallel)](image2)
pair recognizes protonated C and (ii) the 'purine motif' in which the third strand, composed primarily of purine bases, is antiparallel to the central purine strand where AT base pair recognizes A and GC base pair recognizes G. Common features to both these motifs are the necessity of purine bases (A & G) in the central strand.

The triplex formation also depends on backbone composition, structural environment and cation concentration. Different approaches have been adopted in expanding the scope of triplex stabilization. These approaches center on the use of unnatural synthetic heterocycle as bases and chemically modified backbones. Since oligonucleotides do not enter cells very 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 chapter gives an overview of the application of synthetic oligonucleotides. A brief introduction to antisense/antigene/ribozyme based therapeutics is presented. The formation of stable triplex formation using chemically modified oligonucleotides is also discussed with emphasis on base and acyclic backbone modifications. Different physical methods for studying triplexes are also described.

Chapter 2: 5-Amino dU (U*) in the Central Strand of DNA Triplex: Recognition of Purines (A & G) In Third Strand

A necessary feature of the natural base triads for triplex formation is the requirement of a purine (A/G) in the central position, since only these provide sets of two hydrogen bonds, donors/acceptors, in the major groove of the double helix. Pyrimidine bases devoid of this feature are either incompatible or lead to triplexes with low stability. The purpose of this investigation was to engineer pyrimidines to provide extra hydrogen bonding sites in the major groove of the derived WC type duplex, in order to endow dual recognition properties for their placement as central bases in the
triplex triads. The possibility of a pyrimidine derivative, 5-amino-dU (U^*) (I), was explored as the central base of a triplex triad, since it possesses the electronic requirements for simultaneous recognition of the complementary bases of a triad. This chapter demonstrates that 5-amino-2'-deoxyuridine (U^*) is compatible in the central position of the triplex triad X^*U^*:A, where X= A/G/2-aminopurine. The rationale for the design is derived from the Figure 2, which reveals that accommodation of U^* in the central strand
of the established pyrimidine and purine motifs is possible only when the HG strand containing A is parallel \((\text{Ap}^*\text{U}^*:\text{A})\) (Figure 2a) and that with G is antiparallel \((\text{Gap}^*\text{U}^*:\text{A})\) (Figure 2d) to \(\text{U}^*\) in the central strand. The observed selectivity for A can be switched to opposite by 2-aminopurine \((\text{AP}, \text{Figure 2f})\). Chemical synthesis of \(\text{U}^*\) and \(\text{AP}\) oligonucleotides are described. Employing UV-melting experiments, a novel recognition selectivity based on the orientation (parallel/antiparallel) of the third strand purines A, G or \(\text{AP}\) with A in parallel motif \((\text{Ap}^*\text{U}^*:\text{A})\) and G/\(\text{AP}\) in antiparallel motif \((\text{Gap}^*\text{U}^*:\text{A})\) is demonstrated. This is a unique case where engineered molecular recognition leads to successful Hoogsteen type base pairing among two unnatural bases (2-aminopurine and \(\text{U}^*\)) located in the same triad. UV mixing indicated the stoichiometry of binding, while CD spectroscopy confirmed the successful triplex formation.

Chapter 3: 5-Amino dU \((\text{U}^*)\) in the Central Strand of DNA Triplex:

Recognition of Pyrimidines (T & C) in Third Strand

As shown in the previous section, triplexes with modified base \(\text{U}^*\) in the central strand exhibited a remarkable orientation selectivity in the third strand purine (A or G) recognition. In this chapter the modified base, \(\text{U}^*\), was employed to investigate its

Figure 3

(a) \((\text{Cp}^*\text{U}^*:\text{A})\)
(b) \((\text{C}^*\text{U}^*:\text{A})\)
(c) \((\text{T}^*\text{U}^*:\text{A})\)
(d) \((\text{T}^*\text{U}^*:\text{A})\)
recognition in the central strand by pyrimidine in the third strand. For this purpose, the rationale proposed is as shown in Figure 3, which indicates that accommodation of U* in the central strand of the established pyrimidine and purine motifs is possible when the HG strand containing C is parallel (Figure 3a), whereas, T recognition is possible in both motifs (Figure 3c,d). The third strand containing C is accepted only in parallel mode (Cp*U*:A) while, T is recognized in both parallel and antiparallel mode (T,L T-<wbr/>p*U*:A). In case of C, the hysteresis pattern showed sensitivity to pH with less hysteresis at low pH.

The combined results of chapters 2 and 3 expand the lexicon of triplex triads with a recognition motif consisting of pyrimidine in the central strand. The results are discussed with respect to the possible roles of the 5-amino group of dU in triplex formation.

**Chapter 4: Chemical Synthesis of Chiral Acyclic Backbone Incorporated DNA Analogs**

The replacement of 2-deoxy D-ribose moiety of nucleoside by an acyclic chain has led to several analogues, which are resistant to cellular enzymes like nucleases and proteases, although many of these acyclic ODN analogues do not form duplexes of acceptable stability, with natural oligonucleotides.

The objective of the study was chemical synthesis of various substituted chiral acyclic monomers containing 2(R/S)-(N-thymin-1-ylacetyl)-amino-1(R/S)-aryl-1,3 propanediol unit.
The choice of this was dictated by intentions to introduce backbone rigidity into serinol derived acyclic analogues by aryl substitution in the acyclic backbone. For this purpose, 2-amino-1-aryl-1,3-propane diol, a synthetic precursor for the broad spectrum antibiotic chloramphenicol was considered.

The aryl substitution not only restricts the conformational mobility of the acyclic chain, but also introduces a second chiral center in serinol, leading to different stereoisomers. The different stereoisomers of 2-amino-1-aryl-1,3-propane diol were transformed into the desired target phosphoramidite monomers as shown in scheme 1.

In order to get the isomer with the inverted stereochemistry at C1, hydroxyl group at C1 was benzoylated using Mitsunobu conditions. The substitution effect on phenyl ring at C1 was studied by functionalizing the phenyl ring with a nitro group,
on reduction gave the amino group, a very useful intermediate for attachment of various linkers and fluorophores.

All these monomers, with different configurations and substitutions, were transformed into the desired phosphoramidite monomers suitable for incorporation into oligonucleotides.

CHAPTER 5: Biophysical Studies and Applications of Backbone Modified DNA Containing Chiral Acyclic Analogs

In this chapter the synthesis and biophysical studies of oligonucleotides containing chiral acyclic 2(R/S)-(N-thymin-1-ylacetyl)-amino-1(R/S)-aryl-1,3-propanediol unit, in the backbone is described.

The possibilities of incorporating different diastereomers for stereochemical fine-tuning of the ss/ds DNA via duplex/triplex formation were also investigated. Further, different substitutions on the phenyl ring were explored to engineer a better set of analogues. The chiral acyclic backbone was also utilized as handle, for fluorescent labeling by the conjugation of functional ligand pyrene in the oligonucleotides.

The outcome of the studies in the formation of stable double and triple helices involving chiral acyclic moieties in the backbone of the oligonucleotides reveal that DNA duplex and triplex formations are not hampered by insertion of chiral acyclic 2-amino-1-aryl-1,3 propanediol into the regular 2-deoxyribose phosphate backbone of DNA. ODNs with 1S,2S modifications are better than those with 1R,2R. The incorporation of 1S,2S isomer in single site at the 3'-end or 5'-end of oligonucleotides have almost same duplex \( T_m \) as compared to the control. Increasing the number of modification to three at 3'-end slightly destabilized the duplex. In contrast, even a single modification at the center of the duplex effected a large destabilization. Surprisingly,
substitutions on the aryl ring with either nitro or amino group had almost negligible effect on the $T_m$s of triplexes.

Interestingly oligonucleotides containing the different stereoisomers showed similar results, without much change in triplex $T_m$s. A significant outcome of triplex $T_m$ data presented in this chapter is that ODNs having the acyclic chloramphenicol analogues within the normal DNA backbone can still form stable DNA triplexes unlike many ODNs containing acyclic analogues. Insensitivity of $T_m$ to stereochemistry and substitution effect points to tolerance of the different geometries and versatility of the presented backbone in the third strand. This is the first example of the acyclic analogues, when present in third strand, stabilizes DNA triplex.

Enzymatic stability studies of these oligonucleotides with 3'-end modification showed nuclease resistance. The chiral acyclic backbone was employed to derive a fluorescent pyrene analogue for probing DNA complementation and energy transfer between the two strands of DNA duplex.