Abstract

The thesis entitled "Synthesis and Biophysical Evaluation of Cyclohexenyl Nucleic acids and its analogues" has been divided into four chapters.

Chapter 1: Introduction to nucleic acids

Chapter 2: Design and synthesis of cyclohexenyl and α-L-cyclohexenyl nucleosides

Chapter 3: Open chain analogues of cyclohexenyl nucleic acids

Section 3A: Design, synthesis and biophysical evaluation of Open chain analogues of cyclohexenyl nucleic acids

Section 3B: Synthesis of cis and trans modified thrombin binding aptamers and their quadruplex formation study

Chapter 4: 4’-Methoxymethyl threose Nucleic acids

Section 4A: Design, synthesis and biophysical evaluation of 4’-Methoxymethyl threose nucleic acids

Section 4B: Synthesis of 4’-MOM TNA modified thrombin binding aptamer, its quadruplex formation and application as a thrombin inhibitor

Chapter 1: Introduction to nucleic acids

This chapter briefly describes structure of nucleic acids, and applications of synthetic nucleic acids in antisense therapeutics. The potential of modified oligonucleotides to act as antisense agents, that can inhibit the expression of a target gene in a sequence-specific manner. Besides having a specific binding affinity to a complementary target polynucleotide sequence, antisense oligonucleotide (ASON) desirably should meet the requirements for therapeutic purposes, e.g., potency, bioavailability, low toxicity and low cost. Several modifications have been developed over the last two decades to enhance the effectiveness of antisense oligonucleotides, the sites of the modifications include sugar, base and phosphate groups. In some of the modifications sugar- phosphate backbone was modified or the sugar ring was replaced with other heterocyclic or carbocyclic rings as well as acyclic units. Also ribose sugars in DNA and RNA have a preference for either N or S conformation. This conformational state always has an impact on the binding affinities towards DNA/RNA. This conformational change occurs due to the substituents on the sugar
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ring especially 3’ and 2’ substituents. This substituent effect also has been discussed in this Chapter. Along with the duplex binding affinities, recently discovered biologically important quadruplex forming ONs were also reviewed and describing their important applications in therapeutics.

Chapter 2: Design and synthesis of cyclohexenyl and \( \alpha \)-L-cyclohexenyl nucleosides

Six membered cyclic analogues are one of the several other prominent modifications for antisense therapy. The furanose ring of natural nucleoside was replaced by six membered ring which conferred suitable rigidity to antisense constructs. The 1,5-anhydrohexitol nucleic acid (HNA) modification exhibited the selective and strong RNA recognition, yielding the desired A-type double helix but prohibited RNase-H recognition. The double bond was introduced to increase the flexibility allowing adaptation and enzymatic recognition, hopefully preserving the increased affinity for complementary targets.

![Figure 1 Natural nucleic acids and modified analogues.](image)

The cyclohexenyl nucleosides have demonstrated potent antiviral activity and the CeNA oligomers have been shown to mimic the function of RNA with increased enzymatic and chemical stability. The presence of the double bond allows enough flexibility within a CeNA-RNA double helix, to be recognized by RNaseH. CeNA also been shown to be useful in siRNA applications. We designed the stereo isomer of cyclohexene nucleoside named as \( \alpha \)-L-cyclohexenyl nucleoside. The major advantage of \( \alpha \)-L-cyclohexenyl nucleoside is DNA like(S-type) sugar geometry, which is capable of eliciting the RNase-H activity as well as advantages of both \( \alpha \)-L-LNA and CeNA. We proposed a most efficient common synthetic route for synthesis of both cyclohexenyl and \( \alpha \)-L-cyclohexenyl nucleosides (figure 4).
Synthesis of cyclohexenyl and α-L-cyclohexenyl nucleosides:

Synthesis started from Diels-Alder reaction between commercially available 5,5-dimethoxy-1,2,3,4-tetrachlorocyclopentadiene 1 and vinyl acetate at 120 °C yields exclusively endo adduct (±)-2. Acetate was hydrolyzed followed by dehalogenation gave 4. Acidic hydrolysis of ketal 4 resulted the rearranged product. To avoid the rearrangement hydroxyl group converted to benzoate. The Bayer-Villiger’s oxidation of ketone (±)-8 gave two regioisomeric inseparable mixture of lactones (±)-9 and (±)-10 in 7:3 proportion respectively, in high yield.

Scheme 1 Synthesis of the mixture of lactones 9 and 10

Reagents and conditions: (i) vinyl acetate, 120 °C, 5h, 84% (ii)H2SO4, MeOH, 85 °C, 8-10 h, 95% (iii) Na, Liq NH3, THF: EtOH 0.5h, 74%. (iv) BzCl, pyridine, 3h, 93% (v)CH3COOH : H2O (6:1), 120 °C, 4 h, 81% (vi) mCPBA, DCM, 0 °C,5h, 92%, 9:10 (70:30).

Mixture of triol compounds 11&12 obtained by reducing the mixture of lactones, which were further subjected for selective 1,3-diol protection to obtain 13 and 12 which are easily isolable. Allylic hydroxyl group of 13 was oxidized and reduced to get 15.
**Scheme 2** Synthesis of 1,3 diol protected key intermediate 13

Reagents and conditions: (i) LiAlH₄, dry THF, -15 °C, 2h, 72% (ii) PhCH(OMe)₂, PTSA, dry dioxane, rt, 24h, 81% (iii) CrO₃, dry pyridine, Ac₂O, dry DCM, 2h, 92% (iv) NaBH₄, CeCl₃ 7H₂O, MeOH, 3h, 80%.

We have introduced an efficient method for enzymatic resolution with good yields as well as good enantiomeric excess. To resolve the enantiomers, compound (±)-13 was subjected to acylation using vinyl acetate as donor and Candida cylindracea lipase (CCL) to get 16 in excellent yield and high enantiomeric purity.

**Scheme 3** Synthesis of cyclohexenyl thymine nucleoside

One enantiomer was used for the synthesis of cyclohexenyl nucleosides, another was used for α-L-cyclohexenyl nucleosides. In compound 16 acetate group was hydrolyzed and inverted the chiral centre to get enantiopure 15, further subjected for Mitsunobu reaction condition to introduce thymine nucleobase.
**Scheme 4** Synthesis of cyclohexenyl thymine nucleoside

The pure enantiomer 17 was used for synthesis of α-L-cyclohexenyl adenine and guanine nucleoside synthesis.

**Scheme 5** Synthesis of α-L-cyclohexenyl adenine nucleoside

**Scheme 6** Synthesis of α-L-cyclohexenyl guanine nucleoside

**Reagents and conditions:**
(i) Isobutyric anhydride, dry N,N dimethyl acetamide, 150 °C, 2h, 72 %
(ii) 23, DIAD, PPh₃, dry THF, overnight, 34 %
(iii) 80% aq CH₃COOH, 60 °C, 8 h, 68%.
Chapter 3: Open chain analogues of cyclohexenyl nucleic acids

Section 3A: Design, synthesis and biophysical evaluation of Open chain analogues of cyclohexenyl nucleic acids

The evolutionary chemistry with respect to nucleic acids suggested that simple acyclic nucleic acids might be preliminary nucleic acids, which ultimately have evolved as present day carriers of genetic information. To counter the entropic loss in acyclic nucleic acids, an attempt was made by introducing a double bond in the acyclic structure. Incorporation of these acyclic thymidine nucleoside mimics in oligomers was also found to be detrimental to the duplex stability similar to the other acyclic derivatives. We presume that the attachment of nucleobase directly to the double bond in this case may have conferred considerable rigidity, leading to reduced ability of the nucleobase to take part in specific W-C hydrogen bonding.

These modifications prompted us to visualize an open chain ene- nucleic acids in which the nucleobase attachment is to a planar double bonded structure through a methylene group, having same number of atoms in the backbone like natural sugar. This would also have a constraint of double bond unsaturation and act as an acyclic version of cyclohexene nucleic acid (Figure 3).

![Figure 3 CeNA and Proposed cis- and trans-open chain analogs of CeNA](image)

Synthesis of cis and trans thymidine monomers:

We started the synthesis with the mono TBDMS protection of 1,3 dihydroxy acetone. By using simple protections and further wittig-reaction with two carbon ylide resulted the intermediates 4a, 4b, which were isolated carefully and characterized by NOE experiments.
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Scheme 7 Synthesis of key intermediates 7a, 7b

Reagents and conditions (i) TBDMSCl, imidazole, dry DMF (ii) DMTrCl, pyridine, overnight (iii) ethylbromoacetate, PPh₃, Toluene, reflux, 5h (iv) DIBAL-H, dry DCM, -78 °C (v) T-Bz, PPh₃, DIAD, dry dioxane, overnight (vi) TBAF in 1M THF, THF, rt, 2h.

Reduction of ester group followed by thymine base introduction by using Mitsunobu reaction condition and TBDMS deprotection gave 7a, 7b (scheme 7). N³ benzoyl group was deprotected and subjected for amidite reaction to obtain cis and trans thymidine nucleosides (scheme 8)

Scheme 8 Synthesis of trans and cis phosphoramidite monomers

Synthesis of modified oligonucleotides, characterization and UV-\(T_m\) studies
Modified oligonucleotides (Table 1) were synthesized on Bioautomation MM4 DNA synthesizer, using phenoxyacetyl (Pac) protected cyanoethyl phosphoramidites and modified amidite building blocks 9a, 9b.

**Table 1 Modified DNA sequences, their MALDI-TOF mass, UV-Tm measurements**

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>mass cal/obs</th>
<th>UV T_m °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA1</td>
<td>caccattgtcacactcca</td>
<td>5363/5367</td>
<td>63.5 62.7</td>
</tr>
<tr>
<td>DNA1-15T&lt;sup&gt;trans&lt;/sup&gt;</td>
<td>caccattgtacacT&lt;sup&gt;trans&lt;/sup&gt;caca</td>
<td>5347/5342</td>
<td>60.2 -</td>
</tr>
<tr>
<td>DNA1-9T&lt;sup&gt;trans&lt;/sup&gt;</td>
<td>caccattgT&lt;sup&gt;trans&lt;/sup&gt;cacactcca</td>
<td>5347/5347</td>
<td>59.6 61</td>
</tr>
<tr>
<td>DNA1-15T&lt;sup&gt;cis&lt;/sup&gt;</td>
<td>caccattgtacacT&lt;sup&gt;cis&lt;/sup&gt;caca</td>
<td>5347/5344</td>
<td>59.3 59.1</td>
</tr>
<tr>
<td>DNA1-9T&lt;sup&gt;cis&lt;/sup&gt;</td>
<td>caccattgT&lt;sup&gt;cis&lt;/sup&gt;cacactcca</td>
<td>5347/5344</td>
<td>60.8 59.6</td>
</tr>
</tbody>
</table>

<sup>a</sup>UV-T<sub>m</sub> values were measured by using 1µM sequences with 1µM cDNA/cRNA in sodium phosphate buffer (0.01M, pH 7.2) containing 150 mM NaCl and are averages of three independent experiments. (Accuracy is ±0.5 °C). <sup>b</sup>The lower case letters indicate unmodified DNA and upper case indicate modified site.<sup>5</sup>tgagagtgtacagtgt was the complementary DNA sequence. <sup>5</sup>gugagugugacagauugug was the complementary RNA sequence.

**Enzymatic stability studies of modified DNA sequences**

Considering nuclease resistance as an important factor, we examined the 3'-exonuclease sensitivity of unmodified homooligomer t<sub>10</sub> as well as cis and trans modified sequences t<sub>8</sub>T<sup>cis</sup> and t<sub>8</sub>T<sup>trans</sup> by using phosphodiesterase I from *Crotalus adamanteus* venom [snake venom phosphodiesterase (SVPD)].

**Table 2 Oligomers used for 3'-exonuclease degradation study**

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>mass cal/obs</th>
</tr>
</thead>
<tbody>
<tr>
<td>t&lt;sub&gt;10&lt;/sub&gt;</td>
<td>tttttttttttt</td>
<td>2980/2977</td>
</tr>
<tr>
<td>t&lt;sub&gt;8&lt;/sub&gt;T&lt;sup&gt;cis&lt;/sup&gt;</td>
<td>tttttttttttT&lt;sup&gt;cis&lt;/sup&gt;</td>
<td>2964/2961</td>
</tr>
<tr>
<td>t&lt;sub&gt;8&lt;/sub&gt;T&lt;sup&gt;trans&lt;/sup&gt;</td>
<td>tttttttttttT&lt;sup&gt;trans&lt;/sup&gt;</td>
<td>2964/2961</td>
</tr>
</tbody>
</table>

The unmodified single strand DNA sequence t<sub>10</sub> did not show any 3'-exonuclease resistance. The sequence t<sub>8</sub>T<sup>trans</sup> was stable up to 10 min, which is comparatively stable.
than unmodified \( t_{10} \). The sequence \( t_8 T^{\text{cis}} \) was observed to be stable up to 5h, which was clearly, indicated the very high stability under nuclease conditions (Figure 4).

![Graph showing stability of DNA sequences](image)

**Figure 4** SVPD digestion of native DNA \( t_{10} \) and cis,trans modified \( t_{10} \) sequences

**Section 3B: Synthesis of cis and trans open chain analogue modified thrombin binding aptamer, its quadruplex formation**

The **thrombin binding aptamer (TBA)** was discovered in 1992 by *in vitro* selection and found to inhibit fibrin- clot formation with high selectivity and affinity. NMR and X-ray structural study reveals that TBA forms an intramolecular, antiparallel G-quadruplex with chair like conformation. This G-quadruplex consists of two G-quartets connected by three edge wise loops (one central TGT loop and two TT loops). The aptamer interacts with two thrombin molecules, inactivating only one of them. Although several DNA/RNA aptamers are able to show interesting and promising pharmacological properties, rarely they could be used as therapeutic agents without
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modifications due to less stability. To overcome these problems lot of attempts has been done via chemical and structural modifications.

The UNA analogues were used by Wengel and co-workers. These analogues found to be excellent for stabilizing loop structure in aptamers due to its ability to alleviate strain. We studied the flexibility parameter of our open chain-NA modification by introducing it in the loop region of TBA quadruplex.

Table 3 Modified TBA sequences, their MALDI-TOF mass analysis and biophysical evaluation by CD-\(T_m\) measurements

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence(^a)</th>
<th>mass cal/obs</th>
<th>HPLC (t_R) (min)</th>
<th>CD(T_m) (^\circ)C</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBA</td>
<td>ggttggttgttggg</td>
<td>4726/4730</td>
<td>9.8</td>
<td>49.5</td>
</tr>
<tr>
<td>TBA-3T(^{cis})</td>
<td>gg(^T^{cis})tggttgttggg</td>
<td>4710/4709</td>
<td>10.0</td>
<td>38</td>
</tr>
<tr>
<td>TBA-7T(^{cis})</td>
<td>ggttggttggttgttggg</td>
<td>4710/4709</td>
<td>10.1</td>
<td>41.4</td>
</tr>
<tr>
<td>TBA-3T(^{trans})</td>
<td>gg(^T^{trans})tggttgttggg</td>
<td>4710/4714</td>
<td>9.9</td>
<td>36.1</td>
</tr>
<tr>
<td>TBA-7T(^{trans})</td>
<td>ggttggttggttgttggg</td>
<td>4710/4708</td>
<td>10.3</td>
<td>43.7</td>
</tr>
</tbody>
</table>

Figure 5 (a) CD spectra of oligomers TBA, TBA-3T\(^{cis}\), TBA-7T\(^{cis}\), TBA-3T\(^{trans}\), TBA-7T\(^{trans}\) sequences of 5\(\mu\)M concentration in 10mM potassium phosphate buffer (pH 7.5) containing 100mM KCl at 5\(^\circ\)C. (b) Temperature-dependent changes in CD amplitude at 295nm plotted against temperature, first derivative plots at strand concentration 5\(\mu\)M in 10mM potassium phosphate buffer (pH 7.5) containing 100mM KCl.

All four modified sequences showed maxima at 295nm which is characteristic CD signature for formation of stable antiparallel quadruplex (Figure 5a). The stability of the G-quadruplexes was followed by the change in the amplitude of the CD signal at 295nm with temperature. The CD melting results tells that T7 position modifications
are showing less destabilization of antiparallel quadruplex structures as compared to their corresponding T3 position modifications (Figure 5b).

**Duplex stability studies of modified TBA oligomers**

The binding affinity of 15mer TBA ONs TBA, TBA-3T\textsuperscript{cis}, TBA-7T\textsuperscript{cis}, TBA-3T\textsuperscript{trans} and TBA-7T\textsuperscript{trans} with complementary DNA and RNA was also investigated by measuring the melting temperatures (UV $T_m$) of the duplexes (Table 4).

### Table 4 UV $T_m$ (°C)\textsuperscript{a} values of TBA and modified TBA: DNA/RNA duplexes

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence\textsuperscript{b} 5'→3'</th>
<th>mass cal/obs</th>
<th>CDT\textsubscript{m} °C</th>
<th>cDNA\textsuperscript{c}</th>
<th>cRNA\textsuperscript{d}</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBA</td>
<td>ggttggggtggttgtgg</td>
<td>4726/4730</td>
<td>62</td>
<td>63</td>
<td></td>
</tr>
<tr>
<td>TBA-3T\textsuperscript{cis}</td>
<td>ggT\textsuperscript{cis}tggtggttgtgg</td>
<td>4710/4709</td>
<td>54</td>
<td>53</td>
<td></td>
</tr>
<tr>
<td>TBA-7T\textsuperscript{cis}</td>
<td>ggttggT\textsuperscript{cis}tggttgtgg</td>
<td>4710/4709</td>
<td>53</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td>TBA-3T\textsuperscript{trans}</td>
<td>ggT\textsuperscript{trans}tggtggttgtgg</td>
<td>4710/4714</td>
<td>53</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td>TBA-7T\textsuperscript{trans}</td>
<td>ggttggT\textsuperscript{trans}tggttgtgg</td>
<td>4710/4708</td>
<td>56</td>
<td>55</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a}UV-$T_m$ values were measured by using 1μM sequences with 1μM cDNA/cRNA in sodium phosphate buffer (0.01M, pH 7.2) containing 150 mM NaCl and are averages of three independent experiments. (Accuracy is ±0.5 °C). \textsuperscript{b}The lower case letters indicate unmodified DNA and upper case indicate modified site. \textsuperscript{c}5'ccaaccacaccaacc was the complementary DNA sequence. \textsuperscript{d}5'ccaaccacaccaacc was the complementary RNA sequence.

UV $T_m$ results showed that all modified and unmodified sequences were forming stable duplexes with cDNA as well as cRNA. Independent to the nucleoside and position of modifications, $T_m$ values were same for all sequences with cDNA, cRNA (Figure 6). These cis and trans modifications are destabilizing the duplexes compared to unmodified TBA.
Chapter 4: 4’-Methoxymethyl threose Nucleic acids

Section 4A: Design, synthesis and biophysical evaluation of 4’-Methoxymethyl threose nucleic acids

α-L-threose nucleic acid (TNA), was the first synthetic genetic polymer discovered by Eschenmoser and co-workers. Natural five-carbon ribose sugar found in RNA was replaced with an unnatural four-carbon threose sugar in TNA. The successive nucleosidic units were connected through 2’ and 3’ vicinal phosphodiester linkages. Although the number of methods reported for synthesis of L-Threose in the literature, none of them seemed amenable to large scale preparation, either due to expensive starting materials, laborious workup, or poor yields. We designed a straight forward synthetic route with commercially available cheap starting material D-xylose, which will lead us to 4’-Methoxymethyl modified TNA nucleoside (Figure 7). The design of modified TNA was not only for simplifying the synthesis but also to study the hydration effect due to the methoxymethyl group.

D-xylose was converted to 1,2-O-isopropylidene-D-xylofuranose 2 using acetone, conc. H₂SO₄ and Na₂CO₃ in one pot reaction. Mono methylation of primary hydroxyl group in presence of methyl iodide and silver oxide yielded 3. The secondary hydroxyl group was protected with allyloxycarbonyl group to give 4 in very good yield. The acetonide group in 4 was removed and converted into its diacetate 5 by treatment with AcOH and Ac₂O in presence of catalytic amount of H₂SO₄. Compound 5 on treatment with BSA, thymine and TMSOTf under Vorbrüggen conditions afforded exclusively the β-anomer of thymine derivative 6, in good yield. The alloc group was selectively cleaved using Pd(0) to get 7. The free 3’- hydroxyl group was protected as
its DMT derivative using DMTr-Cl in dry DCM and 2,4,6-collidine used as base, to get 8. Compound 8 on ammonolysis gave the free 2'-hydroxyl compound 9. Phosphitylation of the free 2'-hydroxyl with $N$, $N$-diisopropylamino-2-cyanoethylphosphino-chloridite afforded the phosphoramidite monomer 10 (scheme 9).

**Scheme 9** Synthesis of 4'-MOM threose thymine nucleoside

Reagents and conditions  
(i) Acetone, conc $\text{H}_2\text{SO}_4$, $\text{Na}_2\text{CO}_3$  
(ii) Mel, $\text{Ag}_2\text{O}$, dry ACN  
(iii) Alloc-Cl, dry Pyridine, dry DCM, rt, 3h  
(iv) AcOH: $\text{Ac}_2\text{O}$: $\text{H}_2\text{SO}_4$(10:1:0.1), rt, overnight  
(v) Thymine, ACN, BSA, 70°C, TMS-OTf, 0°C, reflux, 3h  
(vi) $\text{PPh}_3$, $\text{Pd(dba)}_2$, Piperidine, DCM, rt, 15min  
(vii) DMTrCl, 2,4,6-collidine, DCM, rt, 24h  
(viii) 2-cyanoethyl-$N,N$-diisopropylchlorophosphine, DIPEA, dry DCM, rt, 1h.

**Synthesis of modified oligonucleotides, characterization, UV-melting studies**

Modified monomer was incorporated in to 18mer DNA sequence and checked the duplex stability with cDNA and cRNA.

**Table 5** Modified DNA sequences, their MALDI-TOF mass analyses and biophysical evaluation by UV-$T_m$ measurements

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence $^{\text{a}}$</th>
<th>mass cal/obs</th>
<th>UV $T_m$ $^{\text{b}}$ $^\circ$C DNA</th>
<th>UV $T_m$ $^{\text{b}}$ $^\circ$C RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA1</td>
<td>caccattgctacacattc</td>
<td>5363/5367</td>
<td>63.5</td>
<td>62.7</td>
</tr>
<tr>
<td>DNA1-15TNA</td>
<td>caccattgctacactcTNA</td>
<td>5393/5393</td>
<td>59.8</td>
<td>61.9</td>
</tr>
</tbody>
</table>

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DNA1-9T\textsuperscript{TNA} caccatt\textsuperscript{TNA}acaetcca 5393/5387 60 62.2

\textsuperscript{a}UV-\textit{T_m} values were measured by using 1\mu M sequences with 1\mu M cDNA/cRNA in sodium phosphate buffer (0.01M, pH 7.2) containing 150 mM NaCl and are averages of three independent experiments. (Accuracy is \pm 0.5 \degree C). The lower case letters indicate unmodified DNA and upper case indicate modified site. \textsuperscript{b}5'tggaattgacaatggtg was the complementary DNA sequence. \textsuperscript{c}5' uggagugacaauggug was the complementary RNA sequence.

Figure 8 (a) Gel pictures of purified sequences and (b) UV melting profiles of modified DNA sequences with cDNA/cRNA

It was observed that modified sequences are forming the stable duplex structures with both complementary DNA as well as complementary RNA. Modified TNA:RNA complexes are more stable than modified TNA:DNA complexes (Figure 8).

Section 4B: Synthesis of 4'- MOM TNA modified thrombin binding aptamer, its quadruplex formation and application as a thrombin inhibitor

Aptamers are rarely used as therapeutic agents due to less stability towards hydrolytic enzymes. Lot of attempts have been done to overcome these problems by introducing chemical and structural modifications. We considered the synthesis of UNA modified TBA and incorporated the 4'-MOM TNA at T7 and T9 positions to study the effect on stability of the quadruplex. TNA is having the 2'-3' backbone and for comparison we also synthesized the TBA sequences with 2'-5' modification at 7\textsuperscript{th} and 9\textsuperscript{th} position and we studied the tetraplex stability, anticoagulation activity as well as enzymatic stability.
Modified TBA sequences were synthesized on automated Bioautomation MM-4 DNA synthesizer and the modified monomers incorporated at 7th and 9th positions. All the sequences purified by HPLC, and masses were confirmed by MALDI-TOF().

**CD spectroscopy and T_m measurement of the TBA sequences in presence of K^+ ion**

The G-quadruplex formation of the modified sequences was studied by CD spectroscopy in the presence of monovalent cation K^+ and their stability was determined as a function of temperature dependent change in CD amplitude at 295nm.

**Table 6** Modified DNA sequences, their MALDI-TOF mass analyses and biophysical evaluation by CD-T_m measurements

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence*</th>
<th>mass cal/obs</th>
<th>CD T_m °C(K^+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBA</td>
<td>ggttgttgtggtgg</td>
<td>4726/4730</td>
<td>49.5</td>
</tr>
<tr>
<td>TBA-7T</td>
<td>gttggtgT^{TNA}gttgtgg</td>
<td>4756/4754</td>
<td>59.7</td>
</tr>
<tr>
<td>TBA-9T</td>
<td>gttggtgT^{TNA}gttgtgg</td>
<td>4756/4754</td>
<td>34.3</td>
</tr>
<tr>
<td>TBA-7T(iso)</td>
<td>gttggtgT^{iso}gttgtgg</td>
<td>4726/4729</td>
<td>46.5</td>
</tr>
<tr>
<td>TBA-9T(iso)</td>
<td>gttggtgT^{iso}gttgtgg</td>
<td>4726/4731</td>
<td>36.5</td>
</tr>
</tbody>
</table>

*The lower case letters indicate unmodified DNA and upper case indicate modified site
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Figure 10 (a) CD spectra of oligomers TBA, TBA-7T, TBA-9T, TBA-7T(iso), TBA-9T(iso) of 5μM concentration in 10mM potassium phosphate buffer (pH 7.5) containing 100mM KCl at 5°C. (b) CD-T_m in 10mM Na-phosphate buffer (pH 7.5) containing 100mM KCl.

Anti-thrombin activity measurements

The anti-thrombin activity of the aptamers on thrombin-catalyzed conversion of fibrinogen to fibrin (clotting) was investigated by measuring the percent transmittance with time. TBA slowed down the coagulation with an increased induction time (t_i as coagulation parameter), confirming its reported inhibitory activity. The induction time for the TBA-7T was higher than for TBA, this high induction time could give a large window to reduce the concentration of TBA in acceptable therapeutic range.

Figure 11 Antithrombin activity measured by % transmittance at 450nm in the presence of TBA and TBA-7T and % transmittance vs wavelength plots at different time-points of the study. ↔ indicates induction time as coagulation parameter (t_i).
Stability of quadruplex structure of aptamers to SVPD

We studied the stability of TBA and TBA-7T quadruplex structures against SVPD enzyme. The stability of TBA-7T was found to be very high compared to the control TBA. The reaction was monitored by change in CD amplitude at 295nm.

Figure 12 Quadruplex stability of the aptamers TBA and TBA-7T (7.5μM) towards Snake venom phosphodiesterase (SVPD) enzyme (2.5 mg/mL).