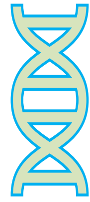


Chapter 7

Conclusion
&
Future Prospects

7.1. Conclusion

Genetic diseases are an implication of deviation from the typical DNA sequence, either in whole or in part. A defective genetic sequence produces a flawed mRNA sequence. Because mRNAs encode for all cellular proteins, drugs targeting faulty mRNAs are thus an optimal choice to interfere directly at the production level before they are decoded to disease causing proteins. RNA therapeutics are a new class of medications designed to bind sequence specifically and target complementarily to their sense-mRNAs, inhibit the production of diseases causing proteins and thereby modulate their gene expressions. Unlike the small drug molecules and monoclonal antibodies, RNA therapeutics are a class of RNA-based synthetic drug macromolecules that target not the proteins but the mutated mRNAs treating the diseases the scientific community considers difficult to be treated by small drug molecules. RNA therapeutics thus have the potential to treat a wide range of diseases, revolutionizing the way treatments are found, developed, and produced on a scale uncommon in the traditional pharmaceutical sector.

Since the discovery of antisense molecules, research into therapeutic use of synthetic Antisense Oligonucleotides (ASOs) to treat various diseases has advanced very quickly. Over the past few years there has been a sharp rise in the number of antisense medications entering into phase III clinical trials as a result of the expanding research on antisense molecules and their processes. Several antisense medications *Fomivirsen, Mipomersen, Eteplirsen, Inotersen, Nusinersen etc.* have received FDA approval for the treatment of breast cancer, cardiovascular disorders, and a variety of infectious and inflammatory diseases. Today, specifically crafted medications based on antisense technology are used to treat a wide range of disorders, many of which cannot be treated with the available traditional pharmacological technologies.

Nevertheless, for successful clinical application, the modified ASOs should possess high resistance to endonucleases, high affinity for target complementary RNA sequences and bind by *Watson-Crick* base-pairing forming ASO/RNA duplexes along with excellent RNA selectivity. The cellular endonuclease RNase H is activated by the ASO/RNA duplexes which then specifically cleaves the RNA strand from the ASO/RNA duplexes in a targeted manner. However, the ASOs are degraded prior to duplexing due to their limited stability in biological media and hence they must undergo thorough chemical alterations to impart a genuine antisense response.

With enhanced thermodynamic stability, nucleic acid recognition, aqueous solubility, superior hybridizing affinity, sequence selectivity and improved bio-stability compared to that of natural oligonucleotides, Locked Nucleic Acids (LNAs) have proven as one of the most efficient members of the antisense modifications. However, some of its kinds were hepatotoxic, comparative nuclease resistance being found to be significantly lower than the PS's and fully modified LNA constructs or with consecutive LNA units resulted in inefficient or sometimes total failure of triplex formation. By optimizing the structural elements of MOEs and LNAs and also by reducing the ASO length (from 20-mer to 14-mer), the potency of MOE based ASOs in animals was increased by 3 to 5-fold ($ED_{50} \approx$ 2-5 mg/kg) without producing hepatotoxicity. Thus, continuous efforts to engineer the LNA structure have resulted in the development of Bridged Nucleic Acids (BNAs) as potent LNA analogues. After all these years of development, the 2′,4′-BNA^{NC} analogues: 2′,4′-BNA^{NC}[NH], 2′,4′-BNA^{NC}[NMe], N-Me-aminooxy BNA and N-MeO-amino BNA modifications have been found to be very promising in antisense technology. Compared to LNAs, the BNAs are highly nuclease resistant, at times even higher than the PSs. Along with higher sequence selective binding affinity for complementary RNAs, stable triplex-forming characters, they were also found to possess excellent single-mismatch discriminating ability. *In-vivo* and *in-vitro* studies showed that compared to the MOE ASOs, optimized BNA ASOs provided increased thermal stability and improved *in-vitro* activity, along with > 5-fold improved *in-vivo* activity. Toxicity parameters like the AST, ALT, liver, kidney, body weights, were also found to be normal for N-MeO-amino BNA, N-Me-aminooxy BNA, and the 2′,4′-BNA^{NC} ASOs. These results suggest that chimeric gapmers with 2′,4′-BNA^{NC}[NH], 2′,4′-BNA^{NC}[NMe], N-Me-aminooxy BNA and N-MeO-amino BNA modifications can be potential drug candidates for applications in the antisense drug discovery platforms.

Interpreting the underlying chemistry of the BNA antisense modifications would help one in understanding their structural and functional significance in exhibiting higher binding affinity with higher hepato-toxicity, mechanism of action as well as guide in postulating superior antisense derivatives. Also, to develop better antisense modifications detailed information on the various existing antisense modifications is very much necessary, which is limited indeed. Quantum chemical studies on a few antisense modifications like cyclohexyl PNA, MOE and LNA have been reported with no such reported study on the titled BNA antisense modifications.

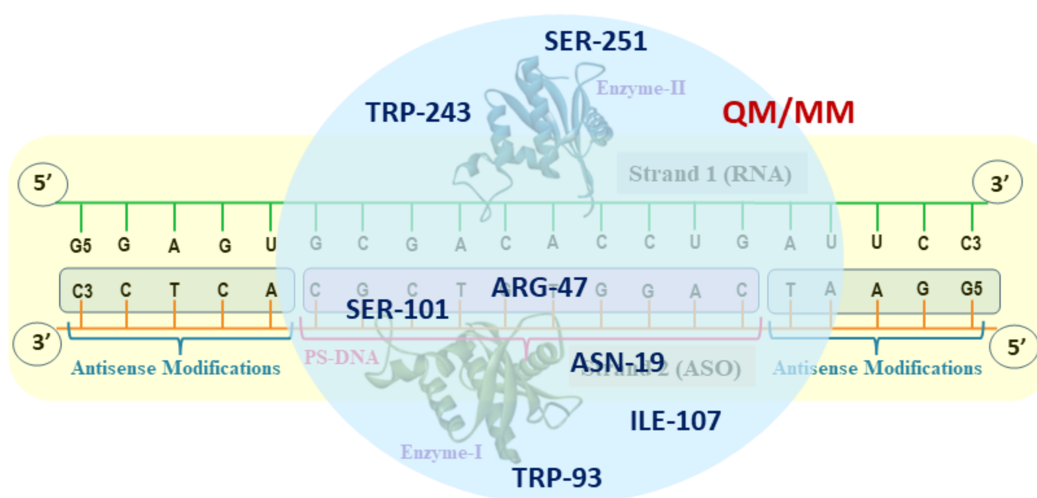
This thesis explored the key features of some of the known as well as a few proposed antisense modifications both at the monomer and oligomer level.

Chapter	Summary
Chapter 1 & 2	▪ Introduction & Review of Literature ▪ Overview of Computational Methods & Tools
Chapter 3	▪ Monomer and Oligomer level investigation of LNA, BNA antisense modifications from literature. ▪ DFT based monomer level studies revealed the various monomer level structural and electronic properties. ▪ 2 sets of MD simulation of 14-mer oligomer duplexes carried out for 1 μs simulation time revealed their various oligomer properties.
Chapter 4	▪ Monomer and Oligomer level investigation of designed antisense modifications A1, A2, A3, A4, A5. ▪ DFT based monomer level studies revealed the various monomer structural and electronic properties. ▪ 1 set of MD simulation of 14-mer oligomer duplexes carried out for 100 ns simulation time revealed their various oligomer properties.
Chapter 5	▪ MD simulation of 20-mer wild type DNA/DNA, RNA/DNA and RNA/RNA duplexes in complex with Human RNase H catalytic domain. ▪ MD simulations of 2 sets, each carried out for 1 μs simulation time. ▪ RNA/DNA duplex had the maximum binding energy with the RNaseH, as expected.
Chapter 6	▪ MD simulation of 20-mer ASO/RNA duplexes in complex with RNase H implementing the designed antisense modifications along with standard PS-DNA, PS-MOE and PS- LNA modifications. ▪ MD simulations of 2 sets, each carried out for 1 μs simulation time. ▪ Comparative study with the wild type duplexes.

7.2. Future Prospects

Despite the exciting prospects, challenges remain on the path to realizing the full potential of RNA therapeutics. Several significant obstacles have to be overcome for the development of RNA therapies, notably the quick exogenous RNA breakdown by tissues and the RNases; transport of negatively charged RNA through cytoplasmic membranes that are hydrophobic; and exogenous RNA with significant immunogenicity that are harmful to cells and prevented the synthesis of therapeutic proteins. Recent developments in RNA biology and bioinformatics have significantly reduced these obstacles, enabling the current rapid development of RNA therapies. Yet, delivery mechanisms, off-target effects, and the need for sustained therapeutic effects pose ongoing hurdles for the researchers and developers. Since the exact mechanism of action of the RNases is not known the present work can be further studied to understand the mechanism of action of various antisense modifications with the RNases, now that, we have some of the common amino acid residues interacting at the active site of the enzyme, apart from DEDD motif residues. Thus, some of our future prospects include:

- QM/MM calculations at the active site of RNase H with amino acid residues ASN-19, ARG-47, TRP-93, SER-101, ILE-107, TRP-243, SER-251.



Scheme 7.1: Schematic representation of QM/MM domain of the modified ASO/RNA hybrid gapmer-type duplexes complexed with dimeric RNase H catalytic domain.

- Explore the two-metal-ion catalysis mechanism, in which two divalent cations, such as Mg^{2+} and $Mn^{2+,}$ directly participate in the catalytic function with the amino acid residues as ASN-19, ARG-47, TRP-93, SER-101, ILE-107, TRP-243, SER-251, obtained from the present studies.