ABSTRACT OF THE THESIS

"Studies on Structure and Dynamics of Bridged Nucleic Acid based ASOs Using Density Functional Theory and Molecular Dynamics Approach for Targeted RNA Therapeutics"

With the discovery of next-generation sequencing, one can now determine the genetic roots of any disease, including Cancer, Huntington's, Parkinson's, Rheumatoid Arthritis, Alzheimer's etc. Although this knowledge has been effectively applied and accepted for diagnosis the science is still lagging behind in terms of pharmaceutical therapies to correct the genetic abnormalities that underlie these diseases. As our understanding of genetics deepens, so does our ability to explore innovative treatments. Gene therapy represents a unique frontier in the treatment of genetic disorders, wherein tailoring therapies to an individual's genetic profile allows for more targeted and effective interventions. Therefore, the current time calls for the creation of medications for personalized genomics.

Over the past century, the biotechnology and pharmaceutical sectors have effectively created techniques to therapeutically target proteins. While there has been considerable progress in creating small-molecule drugs to therapeutically target proteins, RNA based therapeutics provide a more direct approach that alter the RNA function leading to faulty proteins. Using RNA as a therapeutic target has a number of benefits such as the their capacity to interact with targets that are "undruggable" for a protein or small molecule otherwise; their capacity to target a majority of cellular RNAs, their capacity to quickly modify the mRNA construct's sequence in order to create customized treatments or respond to a changing pathogen, the direct translation of genetic findings into drug discovery programs, including the efficiency and speed of the drug discovery process. By targeting specific genetic mutations or variations, RNA therapeutics can address the root causes of genetic diseases with a level of specificity unparalleled by conventional treatments.

As research continues to unravel the complexities of RNA biology, the potential for transformative treatments from traditional supportive measures to cutting-edge precision medicine has offered hope for improved outcomes for individuals affected by genetic disorders. One prominent category of such RNA therapeutics is the use of ASOs. By precisely targeting and modulating the expression of specific genes, ASOs offer a tailored approach to managing disease

conditions at the molecular level. This ability to tailor treatments to an individual's genetic profile opens new avenues for precision medicine. RNA therapeutics thus also holds immense promise in the realm of personalized medicine. 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.

The research work investigates a few BNA antisense modifications both at the molecular and oligomer level. Followed by this, the study aims to design and understand the structural and functional significance of proposed five novel LNA-based antisense modifications labeled as A1, A2, A3, A4, A5 by establishing each with the five standard nucleobases Adenine(A), Guanine(G), Cytosine(C), Thymine(T) and Uracil(U). Since PSs can induce the Human RNase H functions, all the modifications were further implemented with the PS backbone linkage. Oligomer hybrid duplex stability and gene silencing potential of the ASOs are described by performing a detailed classical Molecular Dynamics simulation study on modified ASO/RNA duplexes and duplex gapmers, the ASO strand containing the antisense modifications targeting protein PTEN mRNA sequence of 14-mer in length. This research assisted in comparing the structure-activity relationship of the antisense modifications and development of a systematic DFT & MD method-based paradigm for designing beneficial antisense modifications tuned for specific requirements. The active site of RNases H, known as the DEDD motif contains four negatively charged amino acid residues. The charged residues bind to two metal ions that are required for catalysis. Under physiological conditions these are magnesium ions, but usually manganese also supports enzymatic activity. Based on experimental evidence and computer simulations the enzyme activates a water molecule bound to one of the metal ions with the conserved histidine. The transition state is associative in nature and forms an intermediate with protonated phosphate and deprotonated alkoxide leaving group. The leaving group is protonated via the glutamate. However, the mechanism of the release of the cleaved product is still unresolved. Experimental evidence from time-resolved crystallography of similar nucleases points to a role of a third ion in the reaction recruited to the active site. Therefore, the current research also includes a detailed MD simulation study of 20-mer wild type DNA/DNA, RNA/DNA, RNA/RNA hybrid duplexes including modified ASO/RNA hybrid gapmer-type duplexes containing BNA and the proposed LNA-based antisense modifications in complex with the Human RNase H catalytic domain.

This thesis has been assembled into seven chapters, four of which summarizes the research findings. The conclusion and future prospects of the study are addressed separately in the end of the thesis in chapter seven.

Chapter 1: General Introduction and Review of Literature

Chapter 1 discusses a brief overview of the research subject involving RNA therapeutics and a detailed literature review of the various antisense modifications with a focus on Bridged Nucleic Acid (BNA) based antisense modifications as well as the limitations that have been recognized in the broad area of the research subject. Overall, this chapter describes a concise background of the study, research questions addressed so far and objectives adopted for the current research work.

Chapter 2: Overview of Computational Tools and Methodologies

Chapter 2 discusses very briefly the computational techniques and the tools required to complete the work presented in the thesis. The most popular Density Functional Theory (DFT) under Quantum mechanics (QM) method and Molecular Dynamics (MD) under Molecular Mechanics (MM) method are the major computational methods employed to carry out this research work. Details about level of theories i.e., functionals and basis sets used to calculate the structures and chemical properties are discussed in particulars. Force-field parameterization protocols, charge generation methods and simulation protocols are also mentioned in brief. Additionally, the chapter also lists the software programs utilized to conduct the research work.

Chapter 3: Bridged Nucleic Acid (BNA) ASOs over Locked Nucleic Acid (LNA) ASOs and their impact on the structure and stability of ASO/RNA duplexes.

In **Chapter 3,** monomer nucleotides of bare and phosphorothioate (PS) modified LNA, N-MeO-amino-BNA, 2′,4′-BNA^{NC}[NH], 2′,4′-BNA^{NC}[NMe] and N-Me-aminooxy-BNA antisense modifications were considered for an elaborate DFT based quantum chemical study to estimate their molecular level structural and electronic properties. Oligomer hybrid duplex stability is described by performing a detailed MD simulation study by incorporating the PS-LNA, PS-BNA antisense modifications onto 14-mer ASO/RNA (5′-CTTAGCACTGGCCT-3′/3′-GAAUCGUGACCGGA-5′) duplex gapmers targeting protein PTEN mRNA nucleic acid sequence. Replica sets of MD simulations were performed accounting to two data sets, each simulated for 1 μs simulation time.

Results from both molecular and oligomer level analysis predicted the MO-isosurfaces of the LNA, BNA monomer nucleotides being embedded in the nucleobase region upon duplex formation with target RNAs, the non-RNase H activating segment of the duplexes would be less available for various electron-exchange processes. Also, for the particular nucleic acid sequence solvation of the duplexes although were higher compared to the natural oligonucleotides, but their binding energies being relatively lower may lead to decreased antisense activity. Gapmers modified with LNAs has been reported of potent knockdown activity with hepatotoxic side effects and based on our presumptions the BNA gapmers with similar drawbacks as the LNAs will further keep causing hepatotoxic side effects as LNA gapmers. Hence, fine tuning the existing LNA, BNA antisense modifications to obtain superior binding affinity and higher nuclease resistance is an outmost necessity. This research assisted in forecasting structure-activity relationship of the LNA, BNA antisense modifications and a systematic DFT & MD method-based paradigm for designing advantageous antisense modifications tuned for specific requirements.

Chapter 4: Design of LNA analogues using a combined Density Functional Theory and Molecular Dynamics approach for RNA therapeutics.

In **Chapter 4**, we are proposing five novel LNA analogues (A1-A5) for modifying antisense oligonucleotides and establishing each with the five standard nucleobases Adenine (A), Guanine (G), Cytosine (C), Thymine (T) and Uracil (U), respectively. Monomer nucleotides of these modifications were considered for a detailed DFT based quantum chemical analysis to determine their molecular level structural and electronic properties. A detailed MD simulation study was done on a 14-mer ASO (5′-CTTAGCACTGGCCT-3′) duplex containing the modifications, targeting PTEN mRNA. Results from both molecular and oligomer level analysis clearly depicted LNA level stability of the modifications, the ASO/RNA duplexes maintaining stable Watson-Crick base-pairing preferring RNA-mimicking A-form duplexes. Notably, monomer MO iso-surfaces for both purines and pyrimidines were majorly distributed on the nucleobase region in modifications A1, A2 and in the bridging unit in modifications A3, A4, A5 suggesting A3/RNA, A4/RNA, A5/RNA duplexes to interact more with RNase H and solvent environment. Accordingly, solvation of A3/RNA, A4/RNA, A5/RNA duplexes were higher compared to LNA/RNA, A1/RNA, A2/RNA duplexes. This study has

resulted in a successful archetype for creating advantageous nucleic acid modifications tailored for particular needs, fulfilling a useful purpose to design novel antisense modifications which may overcome the drawbacks and improve the pharmacokinetics of existing LNA antisense modifications.

Chapter 5: A Study on Structure and Dynamics of wild type DNA/DNA, RNA/DNA and RNA/RNA hybrid duplexes in complex with Human RNase H catalytic domain.

In **Chapter 5**, we have performed a detailed MD simulation study of 20-mer DNA/DNA, RNA/RNA, RNA/DNA hybrid duplexes, free human Ribonuclease H (RNase H) enzyme and RNase H bound to the wild type DNA/DNA, RNA/RNA, RNA/DNA duplexes to explore the relationship between the structure and dynamics of these wild type duplexes in complex with RNase H catalytic domain. RNase H is a family of non-sequence-specific endonuclease enzymes with shared substrate specificity for the RNA strand of DNA/RNA duplexes. This research work was performed to understand the mechanism of RNase H in specifically identifying the DNA/RNA hybrid duplex over DNA/DNA and RNA/RNA. Replica sets of MD simulations were performed accounting to two data sets, each simulated for 1 μs simulation time. The trajectories were analyzed for observing structural deviations, duplex stability, inter-strand and intra-strand binding, solvent accessible surface areas and binding affinity with the RNase H. Results from our analysis clearly revealed that for the particular nucleic acid sequence binding of the DNA/RNA hybrid to RNase H to be higher compared to DNA/DNA and RNA/RNA homoduplexes. The active site of nearly all RNases H contains four negatively charged amino acid residues, centered on a conserved sequence motif composed of aspartate and glutamate residues, often also a histidine referred to as the DEDD motif. One significant observation in support of the study revealed the active participation of some common amino acid residues as ASN-19, ARG-47, TRP-93, SER-101, ILE-107, TRP-243, SER-251 which were found to interact with the duplexes for the entire simulation time. This research thus assisted in forecasting the structure-activity relationship of the wild type duplexes in complex with the endonuclease enzyme RNase H and identified some of the important amino acid residues apart from the DEDD motif residues which might play an important role in stabilizing the duplexes at the active site of RNase H.

Chapter 6: A Study on Structure and Dynamics of modified ASO/RNA hybrid gapmer type duplexes in complex with Human RNase H catalytic domain.

In **Chapter 6**, we have performed a detailed MD simulation study of 20-mer PS-DNA/RNA, PS-MOE/RNA, PS-LNA/RNA, PS-A1/RNA, PS-A2/RNA, PS-A3/RNA, PS-A4/RNA, PS-A5/RNA hybrid gapmer-type duplexes to explore the relationship between the structure and dynamics of these duplexes in complex with RNase H in specifically identifying the RNA strand from the modified ASO/RNA duplexes. The study aims to determine the effect of the proposed antisense modifications by our group (A1-A5) with respect to the well-established modifications (PS, MOE, LNA). Because the work focuses on antisense modifications, gapmer type duplexes were considered. Replica sets of MD simulations of free RNase H enzyme and RNase H bound hybrid gapmer duplexes with the different antisense modifications were performed accounting to two data sets, each simulated for 1 μs simulation time. The trajectories were analyzed for observing structural deviations, duplex stability, inter-strand and intra-strand distances and binding affinity with RNase H. Results from our analysis clearly revealed the gapmers consisting of the A3, A4 and A5 modifications are showing strong binding to the RNase H. Other parameters like solvent surface accessible areas and binding energies are also showing favorable effects for these proposed modifications. In continuation of the previous work, compared to the wild type duplexes, HBond analysis of the modified ASO/RNA duplexes revealed the active participation of the same amino acid residues ASN-19, ARG-47, TRP-93, SER-101, ILE-107, TRP-243, SER-251 which were found to interact with the modified ASO/RNA duplexes as well as the wild type duplexes for the entire simulation time. These results assisted in forecasting the structure-activity relationship of modified ASO/RNA duplexes in complex with RNase H and identified the amino acid residues apart from the DEDD motif residues which might play an important role in stabilizing modified ASO/RNA duplexes at the active site of RNase H. This research is thus encouraging to design more novel antisense modifications as such; however, synthesis and experimental validation is necessary.

Chapter 7: Conclusion and Future Prospects

Chapter 7 summarizes the overall illustrative message of the entire work presented in this thesis and makes an effort to incorporate the upcoming research work direction that can be used to improve the understanding and validate the progress of the present research work.

~ Keywords ~

Antisense Oligonucleotides, MOE, PS-DNA, Locked Nucleic Acids (LNA), Bridged Nucleic Acids (BNA), RNase H, ASO/RNA duplexes, Density Functional Theory, Global Reactivity Descriptors, MO Analysis, HOMO, LUMO, Molecular Dynamics Simulations, RMSD, RMSF, RoG, Sugar-pucker, N-glycosidic torsion angle, Inter-Strand and Intra-Strand PP distances, Backbone flexibility, Base-pairing, Base-stacking, HBond, SASA, MM-GBSA.