A biophysical study of small molecule-nucleic acid interactions

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A Biophysical Study of Small Molecule-Nucleic Acid Interactions

by

Rajiv Thapa

A thesis
Submitted to the University at Albany, State University of New York
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the requirements for the Degree of
Master of Science

College of Arts and Sciences
Department of Chemistry
2011
Abstract

Nucleic acids are the biomolecules that encode all the genetic information necessary for cellular metabolism. Binding of small molecules to DNA or RNA can alter their structures; perturb their functions as well as interactions with proteins and other ligands. This thesis focuses on the biophysical study of the interactions of three planar compounds with TAR 32 DNA (32 ntds DNA in HIV-1 LTR, Figure. The purpose for this study is to understand the binding modes of some of the potential drug target compounds, which will be useful for future rational drug design strategies.

Three small compounds interactions with DNA and RNA were investigated using UV-Visible, 1D NMR, Circular Dichroism and Gel electrophoresis. In investigating the small compounds and Nucleic acids interactions, our initial goal is to establish whether the compounds are intercalators or groove binders. UV spectra were collected of the compounds when they were titrated with single stranded and double stranded DNA and RNA, 1D imino proton NMR spectra were used to monitor the titration of compounds into the nucleic acids. Biophysical study showed that the compounds intercalate with nucleic acids, some better than others.

TDP-43 (RNP), which has been identified to play roles in diseases like cystic fibrosis and neurodegenerative diseases, binds to part of the TAR32 DNA sequence used for our study. If compounds bind to TAR32 DNA, possible inhibition of TDP-43 binding to TAR32 DNA is expected. The binding study of the protein with DNA in presence of compound is the topic for future research, which can be useful for future drug design strategies for neurodegenerative diseases.
Acknowledgements

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Lastly, I want to thank my parents, Rama Thapa and Sitaram Thapa, for their love and support. Thanks to my brother (Sanjiv Thapa) and sisters and thank you Manisha, for your love and patience.
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### Abbreviations

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<th>Description</th>
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<tr>
<td>A</td>
<td>adenine</td>
</tr>
<tr>
<td>ALS</td>
<td>Amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>C</td>
<td>cytosine</td>
</tr>
<tr>
<td>CD</td>
<td>circular dichroism</td>
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<tr>
<td>DNA</td>
<td>2’-deoxy ribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EtBr</td>
<td>ethidium bromide</td>
</tr>
<tr>
<td>FTLD</td>
<td>Frontotemporal lobar degeneration</td>
</tr>
<tr>
<td>G</td>
<td>guanine</td>
</tr>
<tr>
<td>HIV-1</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>LTR</td>
<td>long terminal repeat</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
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<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>T</td>
<td>thymine</td>
</tr>
<tr>
<td>TAR</td>
<td>trans-activating response</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-borate-EDTA</td>
</tr>
<tr>
<td>TBM</td>
<td>Tris-borate-magnesium</td>
</tr>
<tr>
<td>TBP</td>
<td>TATA-binding protein</td>
</tr>
<tr>
<td>TDP-43</td>
<td>TAR DNA binding protein</td>
</tr>
<tr>
<td>tRNA</td>
<td>Transfer RNA</td>
</tr>
<tr>
<td>U</td>
<td>uracil</td>
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<tr>
<td>UV</td>
<td>ultraviolet</td>
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Chapter 1: Introduction

1.1. Nucleic acids

The original central dogma of molecular biology stated that genetic information flows from DNA to RNA through the process of transcription and from RNA to proteins through the process of translation (Crick, 1970), Figure 1.1. The central dogma implies that the flow of information is purely unidirectional; however, exceptions to the original central dogma exist. Baltimore and Temin, while studying the reproductive cycle of retroviruses, observed that the genetic information can also flow from RNA to DNA (Temin and Baltimore, 1972). Retroviruses have an RNA genome, instead of a DNA genome, and make a DNA copy of their RNA genome after invasion of a host cell, using the enzyme reverse transcriptase.

\[
\text{transcription} \quad \Rightarrow \quad \text{translation}
\]

\[
\text{DNA} \quad \Rightarrow \quad \text{RNA} \quad \Rightarrow \quad \text{Protein}
\]

\[
\text{reverse transcription}
\]

Figure 1.1. Schematic representation of the central dogma of molecular biology.
DNA in the cell is found in the form of an antiparallel helix of two complementary DNA strands, known as the template (or coding) strand and the non-coding strand. The DNA polymerase enzyme, along with a variety of accessory proteins, catalyzes the replication of both DNA strands using different mechanisms (leading and lagging strand replication) so that the two strands can be synthesized simultaneously. RNA polymerase enzymes (and a variety of transcription factors) facilitate the process of transcription, which generates the RNA product in the 5' to 3' direction with new nucleotides added at the 3' OH group position of the growing RNA chain. The code for protein synthesis is transcribed into the RNA sequence as codons which specify the order of the amino acids in the protein primary sequence. While RNA plays a critical role in the transmission of genetic information directly from DNA to proteins, it is recognized that RNA is more than simply an intermediary in this process (Gilbert, 1986), with non-coding RNA molecules involved in a variety of processes (Matera, Terns, and Terns, 2007). RNA has three direct primary functions in protein synthesis; 1) it serves as the messenger (mRNA) or carrier of the genetic information, 2) it serves as a component (rRNA) of the ribosome, the complex that synthesizes proteins, and 3) it functions as a transfer molecule (tRNA) to transport amino acids, the building blocks of protein.

Ribosomal RNA (rRNA) provides both the structural scaffold and enzymatic function for protein biosynthesis and transfer RNA serves as the raw material carrier in transferring amino acids to the ribosome (Blackburn and Gait, 1996). The ribosome, a ribonucleoprotein (RNP) complex, catalyzes the coupling of amino acids to form the protein primary sequence under the direction of the messenger RNA (mRNA) (Neidle, 2002).
Proteins are a linear biopolymer of 20 different amino acids joined together by peptide bonds. Most of the chemical reactions in living cells are catalyzed and protein enzymes function as the catalyst for these reactions. Proteins also function as structural components of cells. For example, helical assemblies of actin and actin-associated proteins are important for muscle contraction and cytoskeleton (Petsko and Ringe, 2004).

1.2. Nucleic acid structure

In its simplest description, DNA is a linear biopolymer consisting of repeating units called nucleotides, Figure 1.2. The backbone of the polymer chain is a repeating series of sugars connected by phosphodiester bonds. Nucleotides are formed by the linkage of a phosphate group to a nucleoside (nucleosides consist of a nitrogenous base linked to a sugar through a glycosidic bond at the C1' position). In the case of DNA, the backbone sugar is D-2’-deoxyribose and the four most common nitrogenous bases associated with DNA are guanine (G), adenine (A), cytosine (C), and thymine (T). In RNA, the backbone sugar is D-ribose and the most common nitrogenous bases associated with RNA are guanine (G), adenine (A), cytosine (C), and uracil (U).

In canonical Watson-Crick base pairing, adenine-thymine (A-T) or adenine-uracil (A-U) base pairs have two hydrogen bonds compared to three in a guanine-cytosine (G-C) base pairs (Figure 1.4). Watson-Crick base pairing is the canonical base pairing; however, other hydrogen bonded base pairs also exist, with the most common alternatives being Hoogsteen base pairs or base triples and wobbles base pairing. G•U wobble base
pairs have two hydrogen bonds, which results in a reduced stability compared to the Watson-Crick G-C interaction.

Figure 1.2. Schematic representation of single stranded RNA and double stranded DNA. The structures of the nitrogenous bases (C (blue), G (green), A (yellow), U (orange), and T (red)) are shown to the sides and color coded to match the central structure. This figure was taken, without modification, from http://en.wikipedia.org/wiki/File:Difference_DNA_RNA-DE.svg.
DNA can adopt different helical structures that have different global structures and helical parameters, with the most common forms identified as A-DNA, B-DNA and Z-DNA, Figure 1.3. Under physiological conditions, DNA is generally assumed to be in the B-type helical conformation. B-type double helical DNA has 10 base pairs per complete turn of the helix, with the two-polynucleotide chains anti-parallel to each other, and with the A-T and G-C base pairs almost exactly perpendicular to the

Figure 1.3. Three common helical conformations formed by DNA. A-DNA and B-DNA are right handed while Z-DNA is left handed, as shown by the red arrows. The backbone phosphate atoms are indicated in yellow with the attached oxygen in blue. This figure was taken, without modification, from Figure 8.19, (Lehninger, Nelson, and Cox, 2005).
Table 1.1. Summary of the double-helical DNA structural parameters. This table was adapted from Table 6.1, (Mayer, 2010).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>A-Form</th>
<th>B-Form</th>
<th>Z-form</th>
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<tbody>
<tr>
<td>Helical sense</td>
<td>Right-handed</td>
<td>Right-handed</td>
<td>Left-handed</td>
</tr>
<tr>
<td>Base pairs/helical turn</td>
<td>11</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>Major groove</td>
<td>Narrow and deep</td>
<td>Wide and deep</td>
<td>Flattened</td>
</tr>
<tr>
<td>Minor groove</td>
<td>Wide and shallow</td>
<td>Narrow and deep</td>
<td>Narrow and deep</td>
</tr>
<tr>
<td>Major groove width (Å)</td>
<td>2.2</td>
<td>11.6</td>
<td>8.8</td>
</tr>
<tr>
<td>Major groove depth (Å)</td>
<td>13.0</td>
<td>8.5</td>
<td>3.7</td>
</tr>
<tr>
<td>Minor groove width (Å)</td>
<td>11.1</td>
<td>6.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Minor groove depth (Å)</td>
<td>2.6</td>
<td>8.2</td>
<td>13.8</td>
</tr>
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helical axis. B-DNA has a wide major groove and a much narrower minor groove, which has the same depth as the major groove, Table 1.1.

Both A-form and B-form DNA are right-handed, whereas Z-form DNA is left-handed. The bases in the Z-DNA helix alternate between the anti and the syn conformation, which results in a helical repeat consisting of two successive bases with the sugar pucker also alternating between C2’ (anti) endo and C3’ endo. The helix in A-form DNA is wider than the B-form helix with an 11 base pair helical repeat, while Z-form DNA is narrower than B-form, with a 12 base pair helical repeat. The major groove in A-form DNA is very narrow and deep while B-form DNA has a wide and deep major groove and the major groove is flattened in Z-form DNA, Figure 1.3 and Table 1.1. The helical structure of DNA creates the two distinct grooves: the minor and the major, found on the outside of the double strand because of the inherent asymmetry of the N-glycoside connection to the Watson-Crick base pairs, Figure 1.4. In addition, the orientations of
base pairs are such that the major and minor grooves have different functional groups from the nitrogenous bases exposed. In the major groove, A-T and G-C base pairs provide three possible hydrogen bonding sites for other molecules to interact with the DNA: two hydrogen bond acceptors (guanine-N7 and guanine-O4) and one hydrogen bond donor (cytosine-N4) from the G-C base pairs. Similarly, two hydrogen bond acceptors (adenine-N7 and thymine-O4) and one hydrogen bond donor (adenine N6) from A-T base pairs are located in the major groove (Blackburn and Gait, 1996). The minor groove contains the pyrimidine O2 and the purine N3 of the base pair, which serves as a hydrogen bond acceptors. The amino group attached to guanine can serve as a hydrogen bond donor (Neidle, 2002).

**Figure 1.4.** Hydrogen bonding in canonical Watson Crick A-T and G-C base pairs. The major and minor grooves are indicated by orange and yellow curves, respectively. Free hydrogen bond acceptors and donors are indicated in red and blue, respectively. This figure was taken, without modification, from Figure 27.7, (Berg et al. 2002).
Unlike most DNA structures, which are predominantly long stretches of helical duplex, RNA sequences exist as combinations of helical duplex regions, single stranded regions, and a rich diversity of secondary and tertiary structures (Michael and Tor, 1998). RNAs fold back on themselves to form short “hairpins” which have single stranded loop regions inside of double helices formed by Watson-Crick base pairings. The short helices are connected by different types of single strand regions including hairpin loops, bulges, both symmetric and asymmetric internal loops, mismatch loops, as well as pseudoknot structures, Figure 1.5. RNA folds into complex three-dimensional structures that can be recognized by proteins, ligands, and other biomolecules and its biological functions frequently depend on maintaining the proper three-dimensional structures (Hecht, 1996). RNA structures are mostly determined by strong secondary interactions and weaker tertiary structural interactions.
1.3. Nucleic acid-protein interactions

One of the well characterized DNA minor groove binding proteins is the TATA binding protein (TBP) (Kim, Nikolov, and Burley, 1993). Usually, proteins do not bind to the minor groove because it is too narrow, however, TBP has been found to bind to the minor groove with high affinity (Kim et al., 1993). In eukaryotes, RNA polymerases require TBP to initiate the transcription of RNA. TBP is composed of two separate
domains, a 180 amino acid carboxy-terminal domain (highly conserved) and an amino terminal domain that varies in both length and sequence among species. It has been shown that the amino terminal domain is unnecessary for the initiation of transcription. The carboxy-terminal (C-terminal) domain has been shown to bind to TATA-box elements. The C-terminal domain of TBP is composed of two subunits, α and β. Each subunit of TBP consists of a five-stranded, curved, antiparallel β-sheet and two α-helices, which folds into a saddle-like structure. The central eight strands of the curved β-sheet interact with the minor groove of the TATA-element in the DNA (Juo et al., 1996). Upon binding of the TBP, the TATA element is severely distorted, which results in a wide and

Figure 1.6. The structure of TBP-DNA complex. TBP binds to the minor groove of TATA-box DNA, bending the DNA significantly. This figure was taken, without modification, from figure 10-51 (Lodish et al. 1999).
shallow minor groove, more characteristic of A-DNA. The central 8 base pairs of the DNA oligonucleotide that contains the TATA elements unwinds and is bent by 80° toward the major groove to allow for interaction with TBP (Kim and Burley, 1994). The TBP - TATA DNA element complex interaction is dominated by hydrophobic or Van der Waals contacts instead of hydrogen bonding.

Another example of a type of DNA binding protein would be the zinc finger family of proteins. Zinc finger proteins are globular proteins, which have a finger-shaped motif which is used to bind to DNA in a sequence specific manner (Desjarlais and Berg, 1992), Figure 1.7 A. Zinc fingers consist of two anti-parallel β strands followed by an α helix, Figure 1.7A, with cysteine and histidine residues coordinating a single zinc ion within the finger that stabilizes the motif (Luscombe et al., 2000).

The SP1 transcription factor contains a zinc finger protein motif that binds to the DNA. SP1 is a specificity protein (transcription factor), involved in gene expression. Sp1 has 3 zinc fingers, with 2 cysteines and 2 histidines in each finger. Zinc finger proteins of the Cys₂His₂ family are the DNA specific transcription factors (Berg, 1992). Amino acids in the zinc finger tip regions and α-helical regions make base-
Figure 1.7: The zinc finger motif and it’s interaction with DNA. A) A zinc finger motif with the zinc (green) coordinated by two cys residues (S atoms in yellow) and two his residues (N atoms in dark blue) This figure was taken, without modification, from http://en.wikipedia.org/wiki/File:Zinc_finger_rendered.png by (Splettstoesser, 2007). B) Zinc Finger protein (red) bound in major groove of DNA (blue). This figure was taken, without modification from figure 1 A (Nguyen-Hackley et al. 2004).
specific contacts with the major groove of DNA, Figure 1.7B. The zinc finger protein-DNA interaction is determined by the H-bonding interactions between the alpha helix and the DNA segment, mostly between arginine residues and guanine bases.

**1.4. Nucleic acid-small molecule interactions**

Originally, investigations of small molecule-nucleic acid interactions were focused around DNA, rather than RNA, since DNA is both more stable (less subject to degradation) and was easier to chemically synthesize than RNA. For the last two decades, RNA-small molecule interactions have become an active field of research, since RNA synthesis techniques have become easier, less expensive, and more reliable (Ecker and Griffey, 1999). In particular, the targeting of specific RNA molecules for therapeutic agents against viral and bacterial pathogens has become a promising field of investigation (Pearson and Prescott, 1997). In the case of retroviruses, an RNA genome encodes all of the genetic information necessary for replication and maturation of the retroviruses in its host cell environment. Due to the widespread incidence, incurability, and high mortality rates of the human immunodeficiency virus (HIV), efforts to discover drugs to treat or cure the HIV retroviral infection have increased dramatically and RNA studies are at the center of this effort (Balint, 2001). RNA interactions with small molecules have been proposed to be more structure related than sequence selective, compared to DNA-small molecule interactions (Hermann and Patel, 2000). The single stranded regions (loops, bulges, and junctions) distort the neighboring helices and enlarge the major/minor groove with mismatched base pairings and unstacked bases, providing potential binding clefts
and pockets as recognition sites for ligands. The formation of a vast array of complex three-dimensional structures makes RNA-small molecule (ligand) recognition more similar to protein-small molecule interactions.

Nucleic acid (DNA or RNA) - small molecule interactions can be categorized into 3 different groups based on mechanism: 1) non-specific electrostatic binding, 2) intercalation and 3) groove binding, **Figure 1.8.** The shape and functional groups of the small-molecule, the nucleic acid conformation, and the nucleic acid sequence composition largely influence the choice of binding mode. Non-specific electrostatic binding typically involves interactions between the nucleic acid and the ions and water in close proximity to the nucleic acid. These interactions are considered to be non-specific binding interactions between the positively charged ions (e.g. Na\(^+\), K\(^+\) or Mg\(^{2+}\)) and the negatively charged sugar-phosphate backbone of nucleic acids and are an example of typical non-specific electrostatic interactions.

In addition to electrostatic interactions, the binding of a small molecule ligand to a nucleic acid also depends on the shape of the ligand and its complementarity to the shape of the binding site, in order to maximize Van der Waals contacts (Chin et al., 1999). In the case of intercalation, where a planar small molecule ligand is inserted between consecutive base pairs of a nucleic acid helix, perpendicular to the helical axis, the stability of the intercalation complex results from the close packing of the planar molecule between the Watson-Crick base pairs to increase Van der Waals contacts (Palchaudhuri and Hergenrother, 2007). In the case of groove binding, ligands that bind to the major or the minor grooves of nucleic acids make direct hydrogen bonding contacts
with the functional groups of the base pairs.

**Figure 1.8.** Small molecule–nucleic acid interactions. The two strands of the DNA helix are indicated in grey and blue with the various intercalating molecules in red. This figure was taken, without modification, from Figure 8.151, http://www.invitrogen.com/site/us/en/home/References/Molecular-Probes-The-Handbook/Nucleic-Acid-Detection-and-Genomics-Technology/Nucleic-Acid-Stains.html.

Initially, the interactions of nucleic acids with small molecules were investigated using natural dyes. Modified acridines, such as proflavine, were believed to interact with the exterior of the DNA double helix (Lerman, 1961). In 1961, Lerman proposed that proflavine (a planar aromatic molecule) intercalated between the bases of DNA. The publication of Lerman’s hypothesis is considered to be the starting point for the targeting of nucleic acids with small organic molecules. Lerman proposed that there would be two
primary perturbations to the DNA structure upon ligand binding, the DNA length (and viscosity of the DNA solution) would increase and DNA supercoiling would decrease due to helix unwinding. The length of the helix would increase because, around the intercalation site, neighboring base pairs would be separated by approximately 3.4 Å, which lengthens the double helix. Helical unwinding is observed in nucleic acids in order to accommodate the insertion of the intercalator, and this unwinding can be used to verify the mode of small molecule-nucleic acid interaction, since groove binders do not result in helical unwinding (Palchaudhuri and Hergenrother, 2007). Ethidium bromide and proflavine are the most researched intercalating small molecules (Vardevanyan et al. 2003), although there are a number of common small molecule intercalators, Figure 1.9. Simple intercalators consist primarily of fused aromatic rings which mediate binding by insertion between the base pairs. Structurally complex intercalators may also contain positively charged sidechains which interact with the major or minor grooves.

Ethidium bromide (EtBr) is a planar polycyclic molecule that can intercalate between consecutive base pairs of DNA (Figure 1.10). Intercalators including ethidium bromide, often bind to DNA by noncovalent stacking with nucleic acid base pairs. Ethidium bromide is a monocation, when cationic it is an electron acceptor and without a charged alkyl group, it is an electron donor. Ethidium bromide shows large charge delocalization and is a good electron acceptor while AT base pairs are good electron donors (Figure 1.10), which gives favorable conditions for aromatic stacking interactions between EtBr and AT base pairs (Reha et al. 2002). Intercalation of an ethidium bromide molecule into a normal DNA can result in the reduction of helical twist from 36° to 10°,
unwinding the helix by 26°, suggesting that ethidium bromide (EtBr) is a strong intercalator.
Figure 1.9. Simple small molecule intercalators. This figure was taken, without modification, from Figure 1, http://members.localnet.com/~sobell/evidence.html.

Figure 1.10. Ethidium bromide intercalated between A-T base pairs. Atoms are colored grey, blue, red, and white, corresponding to carbon, nitrogen, oxygen and hydrogen, respectively. This figure was taken, without modification, from the abstract figure, (Reha et al., 2002).
Simple intercalators, such as ethidium bromide, shows saturation when the ratio of intercalator to nucleic acid molecule is one molecule of intercalator per two base pairs, which is known as the neighbor exclusion principle. The neighbor exclusion principle states that intercalators can bind to alternating base pairs as potential binding sites (Blackburn et al., 2006). One possible explanation for the exclusion is that binding of an intercalator induces conformational changes at the adjacent sites and thus neighboring sites are sterically unable to bind to an intercalator. Based on the proposed interaction models from Lerman’s Hypothesis, Michael Waring studied small molecule-DNA binding using UV-visible spectroscopy, monitoring the absorbance changes of ethidium bromide upon intercalation into DNA (Waring, 1965). In 1981, the ethidium bromide binding properties were studied with DNA using UV-Visible spectrometric titrations. Ethidium bromide was titrated with Tar32 duplex DNA, ethidium monoazide, and ethidium diazides and the spectral changes monitored, Figure 1.11 (only ethidium bromide data). The UV-Visible spectra showed spectral shifts when DNA was added to free ethidium bromide, characteristic of a classic intercalator. When DNA was added to ethidium bromide, spectral shifts to higher wavelength were seen (red shift) and the spectral intensity decreased as DNA was added in increments. The spectral shift was seen from 476 nm to 516 nm (40 nm).
Figure 1.11. Free ethidium bromide titrated with DNA. A, B, C and D represent increasing concentrations of DNA. This figure was taken, without modification, from Figure 2 (A) (Graves, Watkins, and Yielding, 1981).

While intercalators are usually fused aromatic rings, groove binders are frequently characterized by linked heterocycles that are often crescent shaped (Kutyavin et al., 2003), Figure 1.12. Many groove binders are found to have a preference to bind to A-T rich duplexes and have selectivity for B-form DNA over A-form RNA and DNA (Dervan, 2001). Sterically favorable structures and the large, deep minor groove of DNA are possible explanations for this preference. A-T base pairs can accept hydrogen bonding from the bound molecule at the C-2 carbonyl oxygen of thymine and the N-3
Figure 1.12. Nucleic acid groove-binding small molecules. This figure was taken, without modification, from Figure 8.4.1, (Kutyavin et al., 2003).
nitrogen of adenine. Similar groups are available in G-C base pairs; however, the amino group of guanine creates steric hindrance to forming hydrogen bonding at the N-3 of guanine and O-2 of cytosine. The hydrogen bond between G-C base pairs lies in the minor group, which inhibits small molecule to penetrate the minor groove (Blackburn et al. 2006).

Among the well-known groove binders, distamycin and neotropsin are naturally occurring antibiotics that have been extensively studied and used clinically (Arcomone et al., 1964). In the case of netrospin and distamycin, Figure 1.12, the amide protons hydrogen bond with the adenine N3 and thymine O2 functional groups in the minor groove. In case of netrospin and distamycin binding, the DNA double helix is not extensively elongated or perturbed upon binding, unlike what is seen in intercalating complexes. Minor groove widening (0.5 – 2Å) is usually observed when these simple groove binding small molecules interact with nucleic acids (Bostock-Smith et al., 1999).

1.5. Small molecule inhibition of protein-nucleic acid complexes

TDP-43 is the Trans-Activating Response (TAR) DNA binding protein. TDP-43 is a dimeric protein with two RNA recognition motifs (RRM1 (amino acids 106-175) and RRM2 (amino acids 191-262) and a glycine rich C-terminal domain (Warraich et al., 2010). It was initially identified as a TAR DNA binding protein that acts as a transcription repressor. It was later identified to be a part of a complex that splices the cystic fibrosis trans membrane conductance regulator gene. The TDP-43 functions in the
nervous system are currently unknown, but recent studies have shown that it acts as a neuronal activity response factor and is involved in other neurodegenerative diseases (Mackenzie and Rademakers, 2008). The RNA recognition motifs in TDP-43 bind to the pyrimidine rich motifs of TAR DNA and TG/UG repeats in DNA and RNA oligonucleotides (Kuo et al., 2009).

TDP-43 has been shown to be directly related to the cause of cystic fibrosis, due to exon 9 skipping in the cystic fibrosis trans-membrane conductance regulator gene (Cassel et al., 2010). It regulates the exon 9 skipping by binding to the UG repeats at the 3’ splice site. Recent studies also link TDP-43 with certain neurodegenerative diseases such as frontotemporal lobar degeneration (FTLD) and amyotrophic lateral sclerosis (ALS) (Buratti and Baralle, 2008). Based on its clinical significance, the interaction of TDP-43 with nucleic acids has become a target for small molecule drug intervention and small molecules which inhibit the interaction of this protein with nucleic acids are under investigation. ALS Biopharma has been investigating the roles of TDP-43 in neurodegenerative diseases, as well as the potential drug candidates for these diseases. Recent investigations include the binding of small molecules to TG repeats and TAR32 DNA and screening of small molecules that potentially inhibits TDP-43 binding to nucleic acids (Cassel et al., 2010).

(LTR) sequence (commonly known as the TAR32 DNA) was used for characterization of the TDP-43 protein DNA interactions (Ou et al., 1995). The same TAR32 DNA sequence was also previously used in functional studies by ALS Biopharma.
1.6. Objectives

This project was initiated in collaboration with ALS Biopharma (ALS Biopharma, LLC, 3805 Old Easton Road, Doylestown, PA 18902). The purpose of the project outlined in this thesis was to investigate how a set of specific drug candidate small molecules interact with DNA, the nature of this interaction (intercalation or groove binding), and if this interaction demonstrates either nucleic acid type (DNA vs RNA), structural (single-stranded vs double-stranded), or nucleic acid sequence specificity. While definitive evidence of interactions directly between the small molecule and the DNA target would eliminate these small molecule compounds as TDP-43 targeting drug candidates, it is essential to determine if that is the mechanism of inhibition, and the mode of small molecule-nucleic acid interaction of these compounds was also of interest.

In this study, we have investigated the modes of binding of the small compounds in question to the TAR32 DNA. Presumably, binding of the small molecule to the TAR DNA alters the DNA structure and disrupts the binding of TDP-43. In order to identify the modes of binding of different compounds, we have used UV-Visible spectroscopy, Circular Dichroism (CD) spectroscopy, and 1D proton NMR spectroscopy (Zhang, Hu, and Pan, 2011). UV spectra of the compounds were collected upon titration with single stranded and double stranded DNA and RNA targets. 1D proton NMR spectra were measured in order to detect dimerization and duplex formation as well as to monitor the shifts in imino proton resonances in the nucleic acid targets when titrated with the compounds. Finally, Circular Dichroism (CD) spectra were collected for all of the nucleic acid samples.
2. Materials and Methods

2.1. Nucleic acid sequences

The 32-nucleotide DNA sequence used for this study is derived from nucleotides -18 to +14 of the HIV-1 LTR (Ou et al., 1995). Mutational studies have shown that mutations within this region disrupt the binding of TDP-43 to the HIV-1 LTR (Ou et al. 1995). Mutations were done in pyrimidine rich regions between -15 and -5, between +4 and +11 or in both regions, simultaneously, Figure 2.1. Gel retardation studies demonstrated that the binding of the TDP-43 protein was eliminated by mutations in these pyrimidine rich regions in the HIV-1 LTR (Ou et al., 1995). The TDP-43 protein motif is shown to bind to the pyrimidine rich sequence in HIV-1 DNA (Ou et al. 1995).

![Figure 2.1](image_url)

Wildtype and mutant sequences from the HIV-1 LTR region mutational study. This figure was taken, with modification, from Figure 7, (Ou et al. 1995).

In addition to the TAR32 DNA sequence, several other sequences were investigated in this study, Figure 2.2. The TAR32 complementary sequence

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Figure 2.2. DNA and RNA sequences investigated in this study. The red G nucleotide was added to the TAR32 RNA to promote efficient *in vitro* transcription by T7 RNA polymerase.

(TAR32comp) was used to generate a TAR32 DNA duplex (TAR32:TAR32comp) as well as to check for sequence specificity on the part of the small molecules, since it has the exact opposite sequence compared to the TAR32 DNA, Figure 2.2.
Previous studies have shown that TDP-43 binds DNA more effectively if it has more TG repeats and UG-rich regions (Buratti et al., 2004). Since the binding of the protein to the nucleic acid target increased by almost 100 fold when the number of TG repeats was increased from 3 to 4 (6 to 8 nucleotides) (Cassel et al., 2010), two TG repeat DNA constructs TG3 (TGTGTG) and TG4 (TGTGTGTG) (Figure 2.2) were also investigated. Finally, the TAR32 RNA sequence, which is identical to the TAR32 DNA sequence (with the exception of U nucleotides instead of T nucleotides and the addition of a single 5’ G nucleotide for in vitro transcription), was used to investigate the affinity of the compounds for RNA sequences as well as any differences in affinity for an and the RNA: DNA hybrid duplex formed between the TAR32 RNA and the TAR32 DNA complementary strand (TAR32comp).

2.2. RNA synthesis and purification

The DNA template for the TAR32 RNA (5’- AAC CAC AGA GAC CCA GTA CAG GCA AAA AGC AGC TAT AGT GAG TCG TAT TAG -3’) was ordered from Integrated DNA Technologies (IDT). The RNA used for all of the studies described in this thesis was prepared by in vitro transcription using purified His-tagged T7 RNA polymerase. Briefly, the DNA template was annealed with a T7 promoter strand (5’- CTA ATA CGA CTC ACT ATA G - 3’) to form a partially double-stranded DNA template for in vitro transcription. Small scale test transcriptions (100 µl) were performed to optimize conditions for RNA synthesis. The optimum conditions identified for the transcription were as follows: 40mM MgCl₂, 2.5mM DTT, 4mM NTPs (ATP, CTP, UTP, and GTP),
1x transcription buffer, and 1µM of the annealed T7 promoter: TAR32 RNA template DNA. These optimum conditions were used to run a 30 ml large-scale transcription reaction in a 50 mL falcon tube. The large scale transcription reaction was incubated at 37º C for 7 hours, centrifuged to remove the magnesium pyrophosphate byproduct. 0.1X volume of EDTA (0.5 M, pH 8.0), a chelating agent, was added to the RNA transcription reaction in order to chelate the magnesium remaining in the solution and to stop the reaction. At this point, the RNA was precipitated with 3X volume of cold 100% ethanol overnight at - 20º C.

Following ethanol precipitation, the RNA samples were centrifuged to pellet the RNA, the ethanol was decanted, and the pellet was dried in the hood for 1-2 hours prior to resuspension in sterile distilled deionized nanopure water. RNAs were purified using denaturing polyacrylamide gel electrophoresis (20% 19:1 acrylamide:bisacrylamide 7.8 M urea), in 1x TBE (90 mM Tris:borate, 2 mM EDTA). After electrophoresis, purification gels were backed with plastic wrap and placed on top of fluorescent TLC plates. When the gels were viewed under a UV lamp (short wavelength), the shadow of the RNA band was visible on the TLC plates. Using a clean razor blade, the RNA gel slices were cut out and the RNA was eluted from the gel slices using a Whatman electroeluter (1X TBE). The eluted RNA sample was loaded onto a HiTrap Q column (anion exchange), washed with 10x column volumes of low salt buffer (10 mM sodium phosphate pH 7.6, 1 mM EDTA and 200 mM KCl), eluted with 3x column volumes of high salt buffer (10 mM sodium phosphate pH 7.6, 1 mM EDTA and 1.5 M KCl), and the RNA sample in high salt buffer was precipitated with cold ethanol (3x volume sample
volume) overnight at -20º C. After centrifugation, the ethanol was decanted off the RNA pellets, and the pellets were air dried in the hood followed by resuspension in water. The purified RNA was then desalted and concentrated by ultrafiltration using an Amicon stirred cell. The membrane used for the concentration of RNA had a 1000 molecular weight cut off. The RNA was washed with water three times and then exchanged with phosphate buffer (10 mM phosphate, pH 6.5 with 50 mM KCl) three times. The final concentration of the RNA sample was 1.0 mM. After concentrating the RNA, the sample was stored at -20º C until further use.

2.3. DNA synthesis and purification

The four DNA sequences described above (TAR32, TAR32comp, TG3, and TG4) were ordered from Integrated DNA Technologies (IDT) as 1 µmole scale syntheses, unpurified. The TAR32 DNA and TAR32Comp DNA samples were purified using gel electrophoresis, electroelution, and anion exchange chromatography (Hitrap Q prepacked column), as described in Section 2.2 for the RNA sample. After purification, the samples were desalted and concentrated by ultrafiltration. TAR32 DNA and TAR32Comp DNA were both exchanged and concentrated into phosphate buffer (10 mM phosphate, pH 6.5, 50 mM KCl). The final concentrations of the TAR32 DNA and TAR32Comp DNA were 0.58 mM and 1.0 mM, respectively. Since the sequences were extremely short and the chemical syntheses yielded high quality samples, the TG3 and TG4 DNAs were not purified using gel electrophoresis. The TG3 and TG4 DNAs were subjected to anion exchange chromatography, ethanol precipitated, and then desalted and concentrated into
the same phosphate buffer as the TAR32 DNAs, by ultrafiltration. The final concentrations of the TG3 and TG4 DNAs were 0.7 mM and 0.6 mM, respectively. All DNA samples were stored at -20º C.

2.4. Compounds

The three compounds investigated; ALSB-42771 (CT71), ALSB-42769 (CT-69), and ALSB-42774 (CT-74), are related planar three ring aromatic compounds with varying substituents. The CT69 and CT71 compounds are very similar in structure, with the exception of the replacement of the ethyl group and side-chain primary amino group in CT71 with a methyl ether group and a dimethyl amine group in CT69, respectively. The CT74 compound has the same amine containing side-chain as the CT69 compound, but with a completely different arrangement of functional groups on the benzene ring where the ethyl group (or methyl ether group) are located. ALS Biopharma provided the small molecules used for this study and presentation of the compound structures is restricted, at this point, based on the confidentiality agreement. All of the compounds were obtained as lyophilized powders, dissolved in DMSO (deuterated for NMR studies, non-deuterated for other studies) and the final concentration of all of the stock solution of the compounds were 100 mM. The molecular weights of CT69, CT71 and CT74 are 321,359 and 522 g/mol respectively. Concentrations of compounds were calculated using the molecular weight and the mass of the compound in the tube. Compounds were
dissolved in a microcentrifuge tube and the tube was wrapped with aluminum foil to reduce light exposure and placed in the freezer (-20°C).

2.5. Nucleic acid duplex formation

The TAR32: TAR32comp DNA duplex was prepared in a 1:1 ratio (20 mM TAR32 DNA: 20 mM TAR32comp), heated at 95°C for 4 minutes, and cooled to room temperature. Duplex reactions were tested for time factors ranging from 3 minutes to 6 minutes of heating time; and annealing at 4 minutes the most duplex formation. The mixture was allowed to slowly cool down to room temperature on the bench over an hour. Duplex formation was checked by native gel electrophoresis as described in Section 2.6.

The DNA: RNA hybrid duplex was generated by annealing the TAR32comp DNA and the TAR32 RNA in a 1:1.2 ratio and similarly heated and cooled to room temperature before use, as described above. The reaction was done in a fixed volume and the amount of DNA and RNA mixed together were individually calculated. Duplex formation was tested at different ratios of DNA to RNA. The formation of duplex was investigated using a 15% native polyacrylamide gel at 4°C, as described in Section 2.6.

2.6. Native polyacrylamide gel electrophoresis (PAGE)

Samples were annealed at 95°C for 5 minutes in the presence of 10 mM MgCl₂.

The RNA sample for native PAGE was prepared by mixing the RNA with the 6x loading buffer (30% v/v glycerol in H₂O, 0.25% w/v bromophenol and 0.25% w/v xylene cyanol.
Sample concentration was varied from 50 to 200 nM. Samples were loaded onto native polyacrylamide gels (0.7 mm thick, 20 cm long, 15-20% 19:1 acrylamide- bisacrylamide) run in 90 mM Tris:borate, 10 mM MgCl$_2$ (1x TBM). The duplex samples were first annealed in the presence of 10 mM MgCl$_2$ and then mixed with native loading dye. Gels were run at 4°C and 15 W for 4-6 hours, depending on the RNA, DNA, and DNA duplex sizes, and the percentage of gel used. Gels used in this thesis were stained with SYBR Gold nucleic acid gel stain. Gels were stained for 20 minutes, and gel images were taken using a Biorad ChemiDoc XRS imager and Quantity One 4.6.1 software.

2.7. Compound-Nucleic Acid UV-VIS titration experiments

All three compounds were titrated individually with the various nucleic acid samples. 32 µl of a 500 µM stock solution of the compound was mixed with 768 µl of water to make a working solution of 20 µM compound in the sample cuvette. The reference cuvette was filled with 800 µl of water. All of the spectra were collected using double beam mode and the wavelength range selected for data collection was between 200 and 400 nm. Both cuvettes were placed in the sample compartment and the spectra were collected at room temperature, without temperature regulation. The concentrations of the nucleic acids used for these titrations were between 40 µM and 400 µM. After each titration point was collected, solutions were pipetted out of the cuvette and into an eppendorf microcentrifuge tube and the nucleic acid titrant was added in increments to the sample, as well as the reference sample. Samples were then vortexed, centrifuged briefly, and transferred back to the cuvettes to record the next titration point spectrum.
Depending on the compound and the nucleic acid, the titration ratio of compound: DNA was adjusted from 1: 0 up to 1: 2, by small increments. All UV spectra were collected in 1 cm path length quartz cuvettes. A first sample spectrum was recorded against a reference cuvette that contained 800 µl of water and did not contain DNA. The same amounts of DNA were added to both cuvettes (sample and reference) during the titration. Samples were not annealed and the equilibration time for each titration point was ~ 5 to 7 minutes.

A Cary 300 UV-VIS spectrophotometer equipped with a 6x6 cell block and a temperature controlled sample compartment (running WinUV software version 3.0) was used to collect all of the UV titration data. The titration data, absorbance as a function of wavelength, was saved on the instrument and plotted and analyzed using Kaleidagraph 4.0. The wavelength shift values were determined by zooming in on the spectra and mapping the distance between the peak maxima.

### 2.8. Circular Dichroism (CD) Spectroscopy

The concentrations of the nucleic acid samples used for CD spectroscopy were 3 µM. Spectra were also collected with 6 µM nucleic acids concentration, however, the ellipticity measurements were too high at this concentration. Nucleic acid samples were diluted with phosphate buffer (10 mM phosphate, pH 6.5, 50 mM KCl) and the reference cuvette was filled with 800 µl of phosphate buffer. The sample and reference cuvettes were placed sequentially in a single cell holder and the spectra were recorded. Data were exported to a spreadsheet and later plotted and analyzed in Kaleidagraph 4.0.
For titrations, the sample mixture and the buffer in the reference cuvette were pipetted out of the cuvette into a microcentrifuge tube; compounds were added in increments, vortexed, centrifuged and transferred back to the cuvette. The ratio of DNA: compounds ranged from 1:0 to 1:20. All of the spectra were collected at room temperature, without temperature regulation. Three sets of spectra were collected at all titration points and each titration point had 5 accumulations. Spectra shown in results are the average of the three sets of spectra. Data were collected between 200 and 350 nm with a scanning speed of 100 nm/min, a fixed digital integration time (D.I.T.) of 1 sec, and 5 accumulations per titration point. All of the CD spectra shown in this study were collected on a J-815 circular dichroism spectropolarimeter (Jasco) equipped with a temperature controller and Spectra Manager II software for instrument control, as well as data analysis.

2.9. Nuclear Magnetic Resonance (NMR) Spectroscopy

The nucleic acid concentration of each sample was 0.1 mM and the volume was 450 μl in a standard NMR tube. All samples were prepared in 90% NMR buffer (phosphate buffer from section 2.2) and 10% D₂O. The compounds were dissolved in deuterated DMSO and the concentration of all the compounds used for the titrations were in between 1 mM and 100 mM.

First, 1D spectra of nucleic acid baseline samples were collected with 0.1 mM nucleic acid sample in a glass NMR tube sealed with polyethylene cap and parafilm. Exchangeable imino proton 1D spectra were collected using a 1-1 spin-echo water
suppression scheme (Bax, Sklená, and Sklenar, 1987) After each titration point was collected, solutions were pipetted out of the NMR tube and into an eppendorf microcentrifuge tube and the compound (titrant) was added in increments to the sample. Samples were then vortexed, centrifuged briefly, and transferred back to the NMR tube to record the next titration point NMR spectrum. The titration ratio of compound: DNA was adjusted from 1: 0 up to ~1: 20, by small increments, depending on the specific compound and nucleic acid. All of the spectra collected utilized 1024 scans, a sweep width of 16 ppm, and were collected at 283 K.

All NMR experiments were performed on a Bruker Avance spectrometer operating at a $^1$H frequency of 500 MHz, equipped with a cryoprobe. All spectra were processed using TOPSPIN 2.1 (Bruker, Inc).
3. Results and Discussion

3.1. Verifying duplex formation

In order to verify the formation of duplexes (DNA/DNA and DNA/RNA), and to investigate the dimerization state of individual sequences, 15% native polyacrylamide gel electrophoresis (PAGE) was performed at 4°C in 1x TBM (90 mM Tris:borate 10 mM MgCl$_2$) for the TAR32 DNA and RNA sequences.

Both the TAR32 and TAR32comp DNAs run as a single band, consistent with monomeric 32 nucleotide single-stranded DNAs, compared to a 69 nucleotide single-stranded DNA control, Figure 3.1A (lanes 3, 5, 6). When the two complementary sequences are combined, there is no evidence of either single-strand, and the band on the gel migrates as expected for the TAR32: TAR32comp DNA duplex (lane 4). Although the TAR32 RNA is only one nucleotide longer than the TAR32 DNA and TAR32comp DNA, the TAR32 RNA showed a significantly different mobility on the native gel, Figure 3.1A (lane 2). However, in the presence of excess TAR32comp DNA, all of the RNA is annealed to form an RNA: DNA hybrid duplex, as manifested by the loss of the free RNA band (lane 1). Since, the mobility difference between the DNA: DNA duplex and the DNA: RNA duplex is similar in magnitude to the difference between the free RNA and DNA single strands, this suggests that the mobility difference of the RNA is predominantly due to the change in mass, although the formation of some secondary structure in the free RNA cannot be
Figure 3.1. Native PAGE of DNA:DNA and DNA:RNA duplexes. A) RNA and DNA samples and duplexes. lanes 1: DNA/RNA hybrid, 2: TAR32 RNA, 3: TAR32comp, 4: TAR32 duplex, 5: TAR32 DNA, and 6: a 69 base oligonucleotide used as a marker. B) Different ratios of TAR32DNA:TAR32comp to form DNA duplex. lanes 1: 1:1 ratio, 2: 1:1.25 ratio, 3: 1.25:1, 5:1:1, 6:1:1.25, and 7:1.25:1). Lane 4 is TAR32 RNA used as a marker. M and D to the left of the figures is for monomer and dimer, respectively.
completely discounted.

When the ratio of TAR32 DNA to TAR32comp DNA was investigated, it was clear that an equimolar ratio of the two strands was appropriate for duplex studies. Interestingly, since the TAR32 RNA was used as a marker on this gel, and was loaded at a higher concentration, it became clear that the TAR32 RNA by itself was also able to dimerize, Figure 3.1B. Re-examining the first gel, it is most likely that the observed faint smear at lower mobility is also due to dimer formation, although it was not definitive from the first gel. For these studies, the following nomenclature will be used: duplex indicates the completely complementary double-stranded helices (DNA: DNA and DNA: RNA), and dimer is used to describe the formation of a bimolecular nucleic acid molecule of unknown structure. While there are certainly hydrogen bonded base pairs in the dimer structure, it is not known how many base pairs are formed or how many stacked base pairs could be expected.

3.2. UV-VIS titration experiments

Titrations were performed for all three compounds separately, with each of the DNA and RNA target sequences. The stock concentration of the compounds (CT69, CT71 and CT74) was 100 µM, and 32 µl of a 500 µM stock solution of the compound
Figure 3.2. UV-VIS spectra of the free compounds at 20µM. A) CT69, B) CT71 and C) CT74.
was mixed with 768 µl of water to make a working solution of 20 µM. The sample is transferred to quartz cuvette and 800 µl of water was added into a reference cuvette. All the spectra were collected against a reference cuvette.

**Figure 3.2** shows the absorbance spectra of the individual compounds, CT69, CT71 and CT74. The absorbance spectra for the compounds have different features between 200 and 450 nm. The CT69 compound has two absorption peaks at ~270 nm and 320 nm, with the highest absorption peak at 320 nm. The CT71 compound shows similar peaks at 263 nm and 322 nm, with the highest absorption peak at 263 nm. The CT74 compound also has two peaks at ~245 nm and 329 nm, with the highest absorption peak at ~245 nm. Spectral changes were monitored for all three (CT69, CT71 and CT74) compounds when titrated with each nucleic acid at different concentrations. Since the compounds were titrated with nucleic acids, and nucleic acids have their highest absorption at 260 nm, the absorption bands at ~320 nm were chosen for interpretation to avoid any interference from the nucleic acids, since nucleic acids do not absorb light at 320 nm. Thus titration spectra were recorded between 225 and 400 nm and the spectra were monitored as the nucleic acid concentration was increased.

A representative titration of the CT69 compound with the TAR32 DNA is shown in **Figure 3.3**. Titration points were performed in the range of 1:0 compound: nucleic acid to 1:1 or greater, in some cases. When the ratio of CT69 to DNA was increased, spectral shifts and a decrease in spectral intensity were observed at 320 nm, indicating the binding of CT69 by the TAR32 DNA, and similar to those observed for ethidium bromide titrated with DNA, **Figure 1.11**.
Figure 3.3. Overlayed UV-VIS spectra of CT69 titrated with TAR32 DNA. Each titration point shown in the figure is color coded as shown in the legend on the right indicating the ratio of CT69 to TAR32 DNA.

Figure 3.4. UV-VIS spectra of the CT69 titration with TAR32 DNA at higher compound to DNA ratios. The ratios of CT69:TAR32 DNA of 1: 0, 1:0.35 1: 0.5 and 1:1 are shown in blue, red, and green, respectively.
(Graves, Watkins, and Yielding, 1981). Spectra at higher ratios of CT69 to DNA no longer show the spectral shifts and the changes in spectral intensity, which might suggest saturation in binding. When the ratio of CT69 to TAR32 DNA was 1:0.5, solution turned cloudy and the spectra were recorded after spinning out the sample in a bench-top centrifuge. The recorded spectrum is shown in Figure 3.4. Spectral shifts (red shifts) values are shown in Table 3.1. Red shifts and decreases in spectral intensity when a compound is titrated with nucleic acids are the characteristic features of a nucleic acid intercalating molecule (Arslantas et al., 2007). Based on the UV-VIS absorption spectra, when titrated with TAR32 DNA, CT69 shows intercalating characteristics. This suggests that either the monomeric TAR32 DNA single strand has some neighboring base pairing, providing sites for CT69 to intercalate between base pairs, or, less likely, that the CT69 compound is intercalating between nucleic acid bases that are not base paired.

All three compounds (CT69, CT71 and CT74) showed reduced intensity and a red shift of the 320 nm absorption band when titrated with TAR32 DNA. The red shifts observed for the CT69 compound were nearly twice those observed for CT71 and CT74 when titrated with the TAR32 DNA (Figure 3.5 and Table 3.1). The red shift of CT69 when titrated with TAR32 DNA is ~19 nm at 320 nm, compared to 8 nm and 10 nm for CT71 and CT74, respectively. The compounds were also titrated with the TAR32comp DNA strand to look for any sequence based specificity, Figure 3.6 and Table 3.1. The titration spectra for both the TAR32 DNA and the TAR32comp DNA are similar; the difference is the magnitude of the red shifts. The red shifts for all three compounds were larger for the TAR32 DNA than the shifts observed with the TAR32comp DNA, which
suggests that the compounds bind better to the TAR32 DNA than the TAR32comp DNA. Compound binding might be somewhat sequence specific; TAR32 DNA has 6 TG repeats whereas TAR32 comp does not have any. This difference in shifts suggests that the compounds binding to the nucleic acids might be sequence dependent.

Table 3.1: Summary of the red shifts observed for the compounds in the presence of various nucleic acids.

<table>
<thead>
<tr>
<th>DNA/RNA</th>
<th>CT69 320 nm</th>
<th>CT71 at 322 nm</th>
<th>CT74 at 329 nm</th>
<th>ETBR at 285 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAR32 DNA</td>
<td>19 nm</td>
<td>8 nm</td>
<td>10 nm</td>
<td>N.D.</td>
</tr>
<tr>
<td>TAR32comp</td>
<td>15 nm</td>
<td>6 nm</td>
<td>6 nm</td>
<td>N.D.</td>
</tr>
<tr>
<td>TAR32 RNA</td>
<td>16 nm</td>
<td>11 nm</td>
<td>6 nm</td>
<td>11 nm</td>
</tr>
<tr>
<td>TAR32 Duplex</td>
<td>23 nm</td>
<td>11 nm</td>
<td>9 nm</td>
<td>16 nm</td>
</tr>
<tr>
<td>DNA/RNA Hybrid</td>
<td>19 nm</td>
<td>10 nm</td>
<td>3 nm</td>
<td>N.D.</td>
</tr>
<tr>
<td>TG3 DNA</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>N.D.</td>
</tr>
<tr>
<td>TG4 DNA</td>
<td>3 nm</td>
<td>*</td>
<td>2 nm</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

N.D. : not determined  * no shift observed
Figure 3.5. UV-VIS titration spectra of the compounds with TAR32 DNA. A) CT69 titration with TAR32 DNA, B) CT71 titration with TAR32 DNA, and C) CT74 titration with TAR32 DNA
Figure 3.6. UV-VIS titration of the compounds with TAR32comp DNA. A) CT69 titrated with TAR32comp DNA, B) CT71 titrated with TAR32comp DNA and C) CT74 titrated with TAR32comp DNA.
Figure 3.7. UV-VIS titration spectra of the TG3 DNA with CT69.

The TG3 sequence (TGTGTG) was also titrated with CT69 to see how the short TG-rich single stranded DNA binds to these compounds, Figure 3.7 and Table 3.1. While the UV-VIS titration spectra of TG3 with CT69 does not appear to have a red shift, there is a slight decrease in spectral intensity at 320 nm over a range of compound to DNA ratios (1:0 to 1:2), which demonstrates much larger shifts for the TAR32 sequences. This decrease in intensity suggests that the compound somehow interacts with the DNA but cannot intercalate. Similar results were seen for CT71 and CT74, with no spectral shifts and a slight decrease in spectral intensity. In order to test the presence of additional TG repeats compounds, TG4 (TGTGTGTG) was also titrated with CT69, CT71 and CT74). The TG4 titration (not shown) also show no or very small shifts (Table
which suggests that the binding is of these compounds are low with the single stranded DNA. However, TG4 seems to bind to CT69, CT71 and CT74 better than the TG3 DNA, suggesting that the binding is stronger when the number of TG repeats is increased. Since these DNAs are very short (6 and 8 nucleotides), binding sites for compounds are limited compared to other nucleic acids (~32 nucleotides) used for our study. The TAR32 DNA has 6 individual TG repeats and its complimentary strand does not have any. Both sequences have 32 nucleotides, and thus the same numbers of potential sites, but the red shifts are higher with TAR32 DNA than TAR32comp DNA. This suggests that the binding might either be sequence dependent or that the TAR32 sequence is more prone to structure formation, although why would not be clear.

After investigation of the single-strands, the individual compounds were titrated with the TAR32 DNA: TAR32comp DNA duplex (TAR32 duplex), Figure 3.8. The most notable changes observed on addition of TAR 32 duplex DNA in all three compounds are: 1) the wavelength shifts to longer wavelength (red shift) for all complexes, the shift is larger when CT69 binds to the duplex DNA (Zhang, Hu, and Pan, 2011), while the red shift in case of CT71 and CT74 complex is about the same), and 2) the intensity of the spectra decreased (hypochromicity) in all cases. The hypochromic effect was more obvious in the case of the CT69: TAR32 duplex complex compared to CT71 and CT74.
Figure 3.8. Absorbance titration spectra of A) CT69, B) CT71 and C) CT74 with TAR32 duplex DNA.
It seems that all three compounds bind to DNA in a binding mode that affects the absorption spectroscopy, with the decrease in absorbance and wavelength shift (red shift) indicative of strong binding. A wavelength red-shift and hypochromic effect on addition of DNA is often indicative of stacking interaction due to intercalation between DNA base pairs (Wu et al. 2005). The compound intercalates between base pairs and perturbs the nucleic acid structure. The intensity of the spectra decreases and red shift is seen in all three compounds when they are titrated with TAR32, TAR32comp, and the TAR32 duplex (Figure 3.8).

The compounds, when bound to single stranded DNA (TG3 and TG4), showed no or very small spectral shifts compared to the spectral shifts when longer DNAs bound to compounds (CT69, CT71 and CT74). The spectral shifts combined with the decrease in spectral intensity suggest that compounds are intercalating with TAR32 duplex DNA.

All three molecules were also titrated against the TAR32 RNA, to examine nucleic acid type specificity. Figure 3.9 shows the absorption spectra of the titrations with the TAR32 RNA. The magnitude of the changes were similar to those of the TAR32comp DNA, slightly less than the TAR32 DNA. However, native gel electrophoresis (Figure 3.1) showed that TAR32 RNA dimerizes, thus the interactions of these compounds with the RNA could be either with the single-stranded (and potentially structured) monomeric or dimeric RNA species, so direct comparison of the results is problematic.
Figure 3.9. UV-VIS spectra of A) CT69, B) CT71 and C) CT74 titrated against TAR32 RNA.
Figure 3.10. UV-VIS absorbance titration spectra of A) CT69, B) CT71 and C) CT74 with TAR32 DNA/TAR32 RNA hybrid.
Finally, the three compounds were titrated with a DNA/RNA duplex (hybrid). The absorbance titrations of all three compounds with the DNA/RNA hybrid are shown in Figure 3.10. As expected, a decrease in spectral intensity (hypochromic effect) and the red shift are seen (Kashanian, Khodaei, and Pakravan, 2010). CT69 shows the higher red shift and decreased intensity of spectra when the ratio of compound to RNA: DNA hybrid is increased, followed by CT71, and CT74 shows the lowest red shift. Among the three compounds, the absorption titrations show that CT69 is a better intercalator than CT71 and CT74, with CT74 showing the smallest shifts. As expected, the short single stranded DNAs, TG3 and TG4 didn’t have any significant shifts, which means the compounds didn’t bind, or bound weakly.

Ethidium bromide is one of the best known intercalating small molecule compounds available (Baldini and Varani, 1986). We titrated ethidium bromide with TAR32 duplex and the absorption spectra shows similar shifts and loss of intensity compared to those observed for CT69 when titrated with the TAR32 duplex DNA, Figure 3.11. Ethidium bromide was also titrated with TAR32 RNA and similar results were seen with the red shift with TAR32 RNA lower than that of ethidium bromide with the TAR32 duplex DNA.
Figure 3.11. UV-VIS titration spectra of EtBr with A) TAR32 duplex DNA, B) TAR32 RNA.
3.3 Circular Dichroism titration spectra

Circular Dichroism provides information about the active chiral molecule in solution. Thus, the three compounds (CT699, CT71 and CT74) will give a zero signal unless they are bound to the chiral DNA. CD is one of the most sensitive spectroscopic technique for probing changes in structural complex as a function of concentrations (Kirti K Patel et al. 2002). CD titrations were performed at 3µM duplex DNA concentrations. Single stranded DNA didn’t show any CD spectra. CD spectra for the DNA/DNA duplex shows the characteristics of a B-form DNA, Figure 3.12 (Selvi and Palaniandavar 2002), 1) Negative ellipticity around 244 nm (due to helicity), 2) Positive ellipticity around 275 to 280 nm (due to base stacking), and 3) Positive ellipticity around 220 nm. In the presence of the CT69 compound, Figure 3.13, the increase in

![Figure 3.12](image_url)  
**Figure 3.12.** CD spectrum of the TAR32 duplex DNA.
Figure 3.13: CD titration of CT69, CT71 and CT74 with TAR32 duplex DNA.
the negative band around 244 nm and decrease of the positive band around 275 nm indicates that the compound is intercalated in between base pairs (Ranjbar and Gill, 2009). When CT71 and CT74 were titrated with TAR32 duplexes, Figure 3.13B, C, the changes in the spectra were very small, consistent with the UV-titration data, suggesting that they act as intercalators, but do not perform as well as CT69.

3.4 Nuclear Magnetic Resonance spectroscopy

NMR samples were purified using an Amicon stirred cell (Millipore), concentrated, and exchanged into NMR buffer (10mM phosphate pH 6.5, 50mM KCl). All of the titration studies were performed with nucleic acid concentrations of 0.1 mM (DNA, RNA, and duplexes). All NMR spectra were recorded on a Bruker DRX 500 MHz spectrometer equipped with a CP-TXI cryoprobe. Exchangeable imino proton 1D spectra were collected in NMR buffer and 10 % D$_2$O at 283K. The H$_2$O resonance is suppressed in the sample using a 1-1 spin-echo water suppression scheme. The number of scans for each spectrum was 1024 scans.

Looking at the UV-visible spectroscopy and circular dichroism data, we know that the compounds are intercalating to some extent. To confirm this, we collected 1D imino proton spectra of the DNAs, RNA, and the duplexes and titrated with the individual compounds. Before taking the NMR spectra of the nucleic acids, it was necessary to make sure that none of the compounds showed peaks around the imino
Figure 3.14 shows the 1D NMR spectra of CT69, CT71 and CT74, respectively.
Figure 3.15. NMR spectra of TAR32 duplex DNA titrated with CT69. The green spectrum is DNA by itself, red is 1:0.1, DNA: CT69, and blue is 1:0.5 DNA: CT69.

region, Figure 3.14. Since there were no peaks in the imino proton region for the compounds, compound peaks were not expected to interfere with interpreting the spectra of the nucleic acids.

Some of the characteristics of titration of intercalators into DNA are broadening of the imino peaks and upfield shifts of the A-T and G-C Watson-Crick base pairs near the intercalation (Strekowski, Watson, and Wilson, 1987). When the ratio of DNA : CT69
is 1: 0.5, a decrease in spectrum intensity is observed, but no significant upfield shift, and a slight broadening of peaks was observed, Figure 3.15.

When the ratio of DNA to CT69 increased to 1:1 and 1:2, more broadening of the peaks is visible and a slight upfield shift is seen, Figure 3.16. The broadening of peaks and the upfield shift are more obvious when the ratio of DNA to CT69 increases to 1:5 or 1:10, Figure 3.17. These spectral changes are characteristic of an intercalating molecule (D J Patel 1976). Groove binders usually have downfield shifts in NMR spectra, and nonspecific outside binders usually won’t have any spectral changes in low field spectral regions.

Figure 3.16. Imino proton region of the duplex DNA titrated against CT69. DNA: CT69 ratios are 1:0, 1:1 and 1:2 respectively.
Figure 3.17. Imino proton region of duplex DNA titrated against CT69. DNA: CT69 ratios are 1:0, 1:5 and 1:10. Broadening of peaks and the upfield shifts are seen in the imino region as well as new peaks appearing.

Figure 3.18. CT74 titration against TAR32 DNA duplex. DNA: compound ratio is 1:0, 1:5 and 1:10. The upfield shift and the broadening of peaks are seen. In the case of the TAR32 DNA duplex titration with CT69, we see upfield shifts, which is
a characteristic of intercalators. The NMR titration study shows that the intercalation of
the compound in between the bases occurs, consistent with the observations from UV and
CD titrations. Table 3.2 summarizes the NMR titration data between the compounds and
DNAs, RNA, and duplexes and the data for the NMR titrations are in Appendix section
of this thesis. The NMR studies are consistent with all three compounds having similar
properties with respect to their interactions with nucleic acids. CT69 seems to be a better
intercalator overall, because the shifts and broadening are more visible and also clear
formation of complex is seen with a new peak appearing.

Table 3.2. NMR titration data summary.

<table>
<thead>
<tr>
<th>DNA/RNA</th>
<th>CT69</th>
<th>CT71</th>
<th>CT74</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAR32 DNA</td>
<td>B, U, new peak at 12.5 ppm</td>
<td>B, U, new peak at 12 ppm</td>
<td>B, U</td>
</tr>
<tr>
<td>TAR32 comp</td>
<td>new peak</td>
<td>new peak</td>
<td>new peak</td>
</tr>
<tr>
<td>TAR32 RNA</td>
<td>U</td>
<td>U</td>
<td>B, U</td>
</tr>
<tr>
<td>DNA Duplex</td>
<td>B, U, new peaks at 12 ppm</td>
<td>U, new peak at 12 ppm</td>
<td>B, new peak ~ 12 ppm</td>
</tr>
<tr>
<td>DNA/RNA Hybrid</td>
<td>B, downfield shift</td>
<td>B, downfield shift</td>
<td>B, downfield shift</td>
</tr>
</tbody>
</table>

B – broadening, U – upfield shifts
4. Summary and Future work

In this study, we used spectroscopic techniques to identify the modes of binding of three planar aromatic molecules (CT69, CT71 and CT74). The modes of binding were studied by titrating DNAs, RNA, and duplexes against the small molecules provided by ALS Biopharma. In the UV-visible titrations of duplex DNA, we identified obvious red shifts and also hypochromicity. We also saw the same phenomenon when the three molecules were titrated against the TAR32 RNA. Formation of dimers in the case of TAR32 RNA probably assists in the intercalation of the small molecule. As expected, we saw no red shift and not a significant amount of hypochromicity when the compounds were titrated with single stranded TG repeats. CD spectra of CT69 titrated against the TAR32 duplex DNA showed perturbation in B-form helical DNA. The shifts in positive and negative ellipticities indicate that CT69 is a better intercalator than CT71 and CT74. The NMR spectra collected for the duplexes titrated against CT69 shows obvious upfield shift in the peaks in imino region, characteristic for intercalating molecule. CT74 titrated with TAR32 duplex also showed the same phenomenon.

After analyzing the data we know that the compounds are intercalators, with CT69 being the best intercalator among the three. The next step in this project might be to study these compounds interactions with quadruplexes. Quadruplexes have recently been targeted for therapeutic research and understanding how these small compounds bind to quadruplexes might be useful.

Also, protein binding assays with TAR DNA in the presence of these molecules might give some insight into drug design strategies.
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Press, USA, July 25.


Appendix: NMR titration spectra

**Figure 1.** $^1$H NMR spectra of TAR32 DNA titrated with CT69 A) DNA:CT69 1:0 to 1:5, and B) TAR32 DNA: CT69 1:0 to 1:20. Spectral intensity of DNA decreases as compounds are added in increments, green color coded spectra is DNA by itself.
Figure 2. $^1$H NMR spectra of TAR32 DNA titrated with CT71. DNA: CT71 1:0 to 1:5. Spectral intensity of DNA decreases as compounds are added in increments, green color coded spectra is DNA by itself.
Figure 3. $^1$H NMR spectra of TAR32 DNA titrated with CT74. DNA:CT74 1:0 to 1:5. Spectral intensity of DNA decreases as compounds are added in increments. Green color coded spectra is DNA by itself.
Figure 4. $^1$H NMR spectra of TAR32comp DNA titrated with CT69 A) DNA:CT69 1:0 to 1:1 and B) DNA:CT69 1:0 to 1:5. Spectral intensity of DNA decreases as compounds are added in increments, green color coded spectra is DNA by itself.
Figure 5. $^1$H NMR spectra of TAR32comp DNA titrated with CT71 A) DNA: CT71, 1:0 to 1:1 and B) DNA:CT71, 1:0 to 1:7.5. Spectral intensity of DNA decreases as compounds are added in increments, green color coded spectra is DNA by itself.
Figure 6. $^1$H NMR spectra of TAR32comp DNA titrated with CT74 A) DNA:CT71, 1:0 to 1:1 and B) DNA:CT71, 1:0 to 1:7.5. Spectral intensity of DNA decreases as compounds are added in increments, green color coded spectra is DNA by itself.
Figure 7. NMR spectra of TAR32 duplex DNA titrated with CT69. A)DNA:CT69 1:0 to 1:0.5 and B)DNA:CT69 1:0 to 1:2. Spectral intensity of DNA decreases as compounds are added in increments, green color coded spectra is DNA by itself.
Figure 8. Imino proton region of duplex DNA titrated against CT69. DNA:CT69 1:0 to 1:10. Spectral intensity of DNA decreases as compounds are added in increments, green color coded spectra is DNA by itself.
Figure 9. CT71 titration against TAR32 duplex DNA. DNA:Compound ratio is A) 1:0 to 1:0.5 and B) 1:0 to 1:2. Spectral intensity of DNA decreases as compounds are added in increments, green color coded spectra is DNA by itself.
Figure 10. CT74 titration against TAR32 duplex DNA. DNA:Compound ratio A) DNA:CT69 1:0 to 1:0.5 to B) 1:0 to 1:2. Spectral intensity of DNA decreases as compounds are added in increments, green color coded spectra is DNA by itself.
**Figure 11.** Ct74 titration against TAR32 duplex DNA. DNA:Compound ratio is 1:0 to 1:10. Spectral intensity of DNA decreases as compounds are added in increments, green color coded spectra is DNA by itself.
Figure 11. CT69 titration against TAR32 RNA. RNA:Compound ratio A) RNA:CT69 1:0 to 1:0.5 to B) 1:0 to 1:5. Spectral intensity of DNA decreases as compounds are added in increments, green color coded spectra is DNA by itself.
Figure 12. CT69 titration against TAR32 RNA. RNA:Compound ratio of 1:0 to 1:20. Spectral intensity of DNA decreases as compounds are added in increments, green color coded spectra is DNA by itself.
Figure 13. CT71 titration against TAR32 RNA. RNA:Compound ratio A) RNA:CT69 1:0 to 1:0.5 to B) 1:0 to 1:5. Spectral intensity of DNA decreases as compounds are added in increments, green color coded spectra is DNA by itself.
Figure 14. CT71 titration against TAR32 RNA. RNA:Compound ratio of 1:0 to 1:20. Spectral intensity of DNA decreases as compounds are added in increments, green color coded spectra is DNA by itself.
Figure 15. CT74 titration against TAR32 RNA. RNA:Compound ratio A) RNA:CT69 1:0 to 1:0.5 to B) 1:0 to 1:5. Spectral intensity of DNA decreases as compounds are added in increments, green color coded spectra is DNA by itself.
Figure 16. CT74 titration against TAR32 RNA. RNA:Compound ratio of 1:0 to 1:20. Spectral intensity of DNA decreases as compounds are added in increments, green color coded spectra is DNA by itself.
Figure 17. CT69 titration against TAR32 hybrid. Hybrid:Compound ratio A) Hybrid:CT69 1:0 to 1:0.5 to B) 1:0 to 1:5. Spectral intensity of DNA decreases as compounds are added in increments.
Figure 18. CT71 titration against TAR32 hybrid. Hybrid:Compound ratio A) Hybrid:CT71 1:0 to 1:0.5 to B) 1:0 to 1:5, green color coded spectra is DNA by itself.
Figure 19. CT74 titration against TAR32 hybrid. Hybrid:Compound ratio A) Hybrid:CT74 1:0 to 1:0.5 to B) 1:0 to 1:5. Spectral intensity of DNA decreases as compounds are added in increments, green color coded spectra is DNA by itself.