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The University of Southern Mississippi

THE USE OF A SMALL MOLECULE TO IMPROVE

THE THERMOSTABILITY OF DNA JUNCTIONS

by

Arik Shams

A Thesis
Submitted to the Honors College of
The University of Southern Mississippi
in Partial Fulfillment
of the Requirements for the Degree of
Bachelor of Science
in the Department of Chemistry and Biochemistry

May 2014

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ABSTRACT

The short-term goal of this research project is to employ small molecules as a means to stabilize four-way DNA junctions (4WJs) composed of natural DNA and chimeric nucleic acids. The long-term goal of the project is utilizing the 4WJs as extracellular therapeutic inhibitors of DNA binding proteins [i.e. Histones and High Mobility Group Protein B (HMGB1b)]. A number of studies have shown that classical intracellular DNA-binding proteins have a variety of deleterious side-effects when present in the extracellular milieu. In order to develop a successful 4WJ therapeutic, we are focused on using modified nucleic acids to enhance the stability of the resulting 4WJ. The nucleic acid of interest is PNA (peptide nucleic acid). PNA was selected because it is known to form DNA-PNA duplex/triplex structures with elevated thermo- and nuclease stability. 4WJs are prepared using fluorescently labeled DNA strands and a single PNA strand. Small molecules are currently being investigated as tools to potentially link the PNA-DNA strands to form 4WJs composed of multiple PNA strands. One molecule of interest is $[Ru(bpy)_2(dpp)PtCl_2]Cl_2$. Electrophoretic mobility shift assays (EMSAs) have shown that stable 4WJs form in the presence of this molecule. The junctions were visualized using polyacrylamide gels. Circular dichroism studies will be employed to characterize the structural properties of hybrid of interest. Once a stable 4WJ structure is identified, the hybrid was used to study binding and inhibition of HMGB1 in cell-based assays.

Keywords: DNA junctions, Holliday junctions, high mobility group proteins, HMGB1, peptide nucleic acids, PNA, moonlighting proteins

DEDICATION

This thesis is dedicated to my lab partner and friend Ann Marie Brahan, my parents

Tawhid Shams Haque and Nazveen Begum, my sister Tahseen Shams, and lastly my

beloved pet fish, Mr. Fish.

ACKNOWLEDGEMENTS

I would like to recognize Dr. Anthony Bell for his unwavering support and guidance during my time in his laboratory. His mentorship went far beyond this particular study, and I remain grateful for his dedication to see me succeed as a student and individual. In addition, the Bell lab graduate students Doug Iverson and Crystal Serrano were also incredibly supportive in the process of completing this research.

I would also like to thank the Honors College for their constant support throughout my four years at The University of Southern Mississippi. I especially thank Dr. Dave Davies, Paula Mathis, and Stacey Ready for their hard work and commitment towards my success. In addition, the entire Honors College staff has been instrumental in my total college experience.

Additionally, I would like to thank the Department of Chemistry and Biochemistry for their support in completing this project. The chemistry faculty has taught me a great deal, including the value of research and academic excellence.

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CHAPTER I – INTRODUCTION

The purpose of my project is to use small molecules (with a molecular weight of 300-600) to stabilize the formation of four-way DNA junctions, and subsequently use those junctions as an extracellular ligand against the proinflammatory cytokine HMGB1 (High Mobility Group B1). DNA four-way junctions (4WJs) are also referred to as Holliday junctions. 4WJs are named after Robin Holliday who proposed that genetic recombination and repair events were mediated by four stands of DNA converging to form a junction[1]. Figure 1 displays a schematic of the role of 4WJs in recombination. Genetic recombination, the primary mechanism of genetic diversity in organisms, takes place when strands of DNA break and rejoin with other strands to produce new DNA strands consisting of new sequences of base pairs[2]. As illustrated in Figure 1, two double-stranded DNA duplexes are recombined to form two new duplexes of the DNA, composed of sections of both the original strands. The crossover region is called the DNA junction. The 4WJ is an intermediate structure, and is naturally unstable during genetic recombination[3]. At the end of recombination, each DNA junction is resolved by the action of enzymes and proteins. The resulting DNA strands are composed of portions of their original sequence and portions of the strand of DNA that it overlapped or combined with[1,3]. The short-term goal of my research project is to utilize small molecules as a means to stabilize immobilized 4WJs composed of natural (DNA) and synthetic nucleic acids such as peptide nucleic acids (PNAs). The long-term goal of the
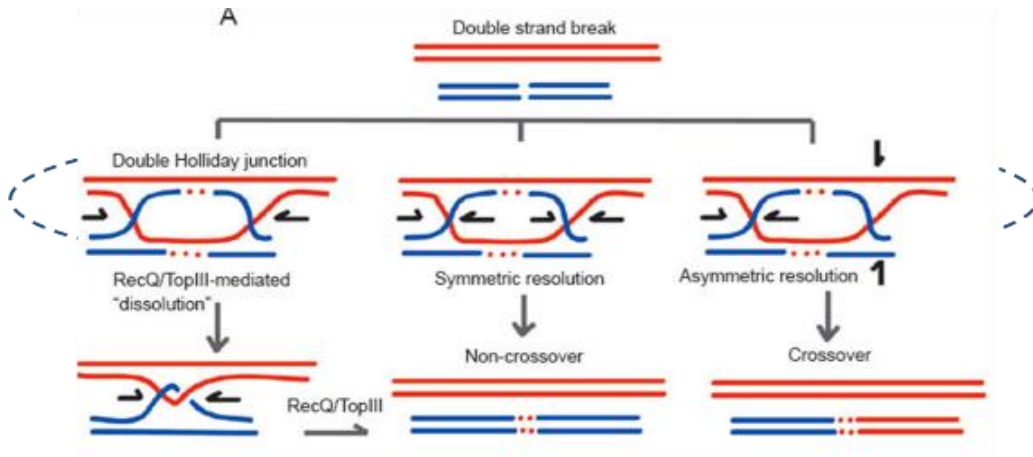
Figure 1: Genetic Recombination with 4WJs
[http://www.nature.com/cr/journal/v20/n6/images/cr201073f1.jpg]

project is to utilize the resulting 4WJs as

therapeutic inhibitors against the

proinflammatory cytokine HMGB1. HMGB1

was originally discovered in the early 70's.

HMGB1 is a highly abundant (10^6

copies per cell) protein found in almost all

cells. HMGB1 is composed of 215 amino

Figure 2: Open-x form of immobilized 4WJ J1, with the four component strands labeled 101, 102, 103, and 104. The 101 strand is labeled with a fluorophore on the 3' end.

acids[4,5]. The protein possesses two DNA binding domains (the a-box and b-box)

followed by stretch of aspartic acid and glutamic acid residues. A schematic for HMGB1

is displayed in Figure 3. HMGB1 is very unique in that it is classified as a "Moonlighting

Protein". As the name suggests, the protein has two different functions. HMGB1 can

function inside of the cell, where it binds/bends DNA to promote transcription. HMGB1

Figure 3: HMGB1. The domain structure is shown in (a), and a 3D schematic of the compact form is shown in equilibrium with the more open form in (b).[5]

can also function outside of the cell, where it binds to immune receptors, such as

Receptors for Advanced Glycation Endproducts (RAGE) to mediate the production of

leukocytes (i.e. amplify the proinflammatory immune response).

The protein was originally classified as an architectural DNA-binding protein whose sole

function was to bind and bend DNA[4,6]. Later it was shown that the protein can be either

actively or passively released from cells into the extracellular matrix where it may bind

to immune receptors. In its alternative capacity, HMGB1 binding offers homeostatic

benefits such as wound healing, dendrite maturation and neurite outgrowth. However,

the abundance of the protein often presents a problem because large amounts of the

protein is often present in the extracellular region where it then binds to trigger

superfluous leukocyte production. As a result, there is often a great deal of collateral

damage to healthy cells and tissues. Hence it is not surprising that HMGB1 has been

linked to a myriad of disease states that include cancer, arthritis, atherosclerosis, lupus,

and sepsis[4].

Our strategy to mediate the deleterious behavior of HMGB1 is centered on combining or reassigning the original function of the molecule. The hypothesis is to reassign hybrid 4WJs to the extracellular matrix where they can act as competitive inhibitors that bind HMGB1. The 4WJs will effectively sequester HMGB1 and prevent its interaction with immune receptors, an outcome that would eventually stop the production of leukocytes. The premise for the strategy is based on the fact there is experimental data that clearly show that HMGB1 binds 4WJ and bent DNA with very high affinity[7,8]. This suggests that these nucleic acid ligands would effectively target HMGB1 extracellularly. One area of concern is the high likelihood that DNA junctions would be rapidly degraded *in vivo*. To offset this, researchers at the University of Southern Mississippi have developed novel hybrid 4WJs composed of DNA and PNA (peptide nucleic acids). PNAs are synthetic hybrids of nucleic acids (DNA and RNA) and peptides that have been shown to possess enhanced stability (both *in vitro* and *in vivo*) compared to conventional nucleic acids[9,10].

Research[10] has shown that 4WJs composed of single PNA strands can be synthesized, and that they form stable structures that are bound by HMGB1b and the DNA-binding protein, Histone H1 with high affinity. However, 4WJs composed of two PNA strands are less stable. In order to increase the stability of these chimeric 4WJs, a series of small molecules are currently being investigated. The structure of a fully-formed compound/4WJ combination *in vitro* will be elucidated, and the resulting junction will be further tested for nuclease and thermal stability.

CHAPTER II – LITERATURE REVIEW

High mobility group B1 protein (HMGB1)

The target protein in this study is high mobility group box 1, or HMGB1. HMGB1 is a small protein comprised of 215 amino acid residues. It is composed of two domains, the A box and the B box, with a short linker between the two, and a long (30 amino acid) acidic C-terminal tail[5] (Figure 3). HMGB1 binds to the minor groove of DNA, and therefore the minor groove of Holliday junctions as well. As HMGB1 is bound tightly to chromatin within the cell, its release into the extracellular matrix is usually a result of necrosis (cell collapse and death due to damage or disease), although active release by apoptotic cells (cells programmed to die at the end of their life cycle) can also occur[11,12]. The cytokine activity of HMGB1 is mostly due to its binding with RAGE, or Receptor for Advanced Glycation End-products. RAGE is a receptor that activates the immune system when it interacts with cytokines such as HMGB1[4,12,13] (Figure 6).

Intracellularly, HMGB1 is a DNA binding protein that favors bent DNA structures. On the extracellular level HMGB1 acts as a pro-inflammatory cytokine. The sequence specificity of HMGB1 to DNA junctions is relatively low, which is due to the fact that HMG1 proteins bind to DNA via contact with the minor groove of the junction rather than the major groove where most transcription factors usually tend to bind[5,7].
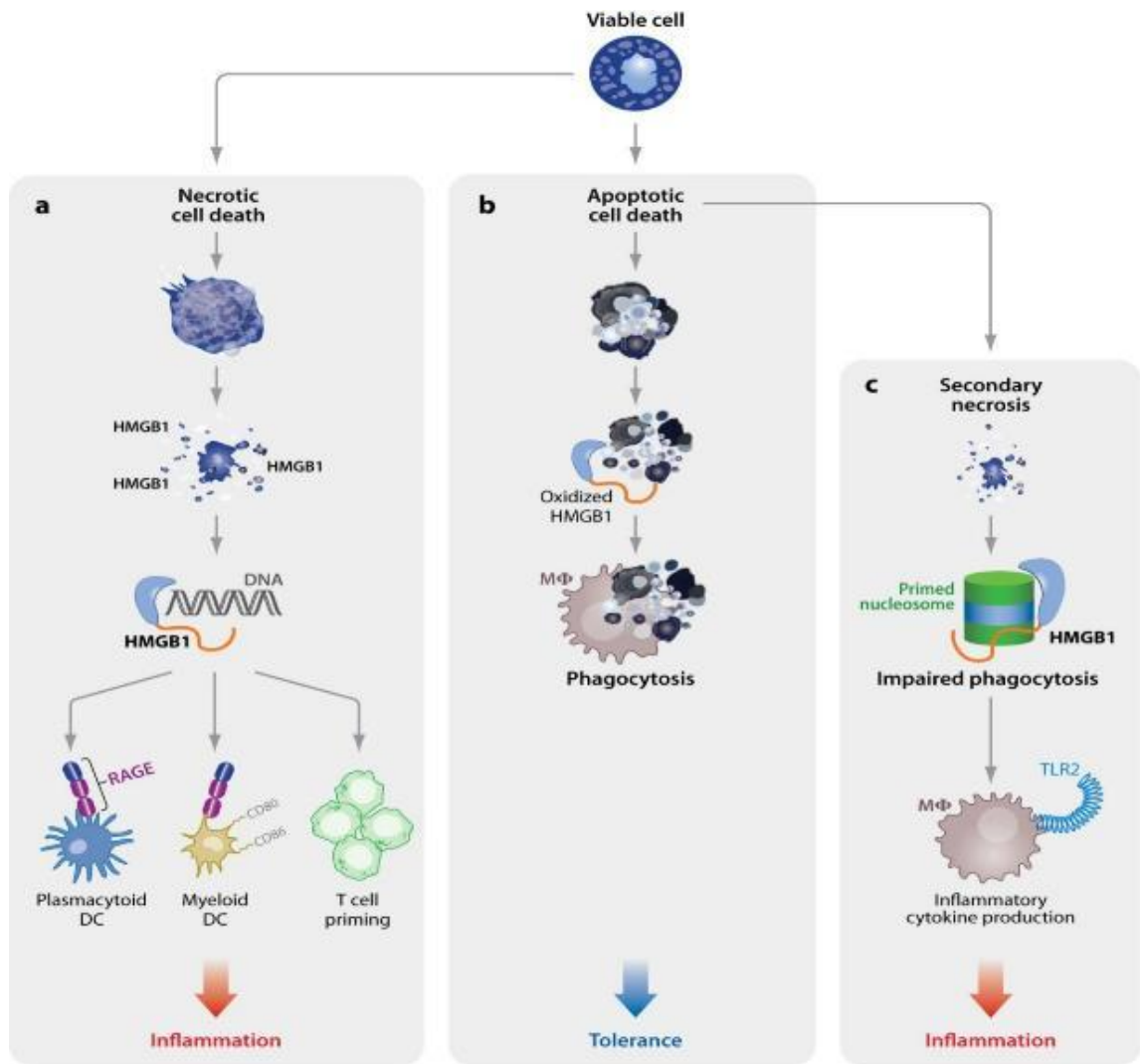
Figure 4: The nature of cell death determines the type of immune response. A) Necrosis is when the cell collapses due to damage or disease and all the cell's internal components are released into the extracellular matrix. In (B), apoptosis is the programmed cell death that results from homeostasis, and is the destruction of the cell at the end of its life cycle. C) Secondary necrosis occurs when there is an error in recognizing apoptotic cell death and the result is pro-inflammatory, like that of (A).[4]

DNA junctions

Four-way DNA junctions (4WJ) are three-dimensional higher-order DNA structures that are intermediates for DNA recombination in the cell. They also act as substrates for protein binding[14]. Proteins that recognize and bind to 4WJ do so at the level of their tertiary structure. During recombination, two DNA oligonucleotides join together at a specific point, where one strand from each strand remains continuous in one axis, while the complementary strands of each DNA molecule fold at the point of joining of the two molecules[1–3] (Figure 1). The basic structure of a 4WJ is outlined in Figure 5. In cells, the 4WJ undergoes branch migration via the activity of the RuvA protein complex[3]. The junction is eventually resolved by the action of exonucleases.

Figure 5: The stacked X-structure of 4WJ. The ribbon indicates the path of the backbones in the right-handed, antiparallel stacked X-structure. The two sides of the structure are not equivalent. One side (m) presents minor groove edges of the base-pairs at the point of strand exchange, while at the other (M) the major groove edges are presented.[14]

4WJ has two major conformations, dependent on cation activity. In the absence of metal cations, 4WJ adopts an open, unfolded structure. Upon the addition of metal cations (most prominently magnesium ions), the double-stranded or duplex arms of the 4WJ stack coaxially[15,16]. This conformational isomer is referred to as the stacked-x structure (Figure 4). The reason for this is that DNA is a charged polyelectrolyte (polymers composed of repeating electrolytic units) that can induce repulsive interactions with metal cations[1,2]. Multiple studies have provided evidence for this observation. As displayed in Figure 5, the structure of stacked-x and open-x 4WJs are very dynamic. At cation concentrations ≤ 0.1 mM the conformation of 4WJs can readily interchange between open-x and stacked-x. The open-x isomer is denoted below in Figure 6 (open-x = b and stacked-x = c)[17]. Figure 7 shows another diagram for the two conformations of 4WJ.
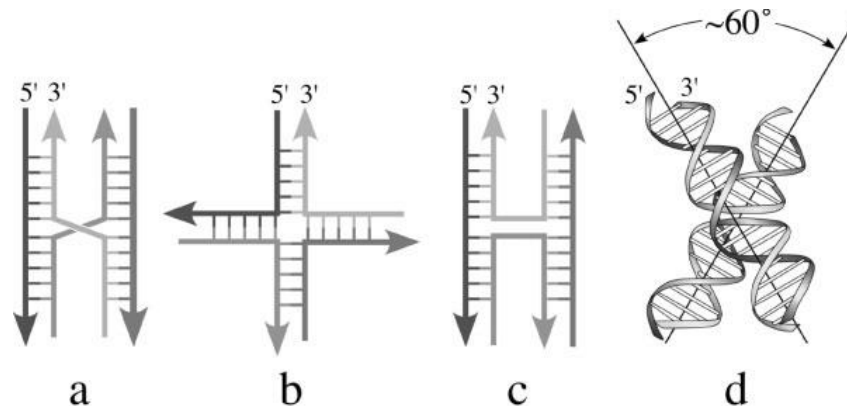
Figure 6: Structural forms of DNA Holliday junctions. a. The parallel stacked-X junction initially proposed by Holliday as the recombination intermediate (Holliday, 1964). b. The extended open- X form of a DNA junction. c. The antiparallel stacked-X junction does not allow for migration of the junction along the DNA strands. d. Model of the antiparallel stacked-X junction proposed from solution studies.[15]
Duckett et al., 1988, copied from Khuu, et. al. J. Mol. Recognit. (2006)

major groove side   face   minor groove side
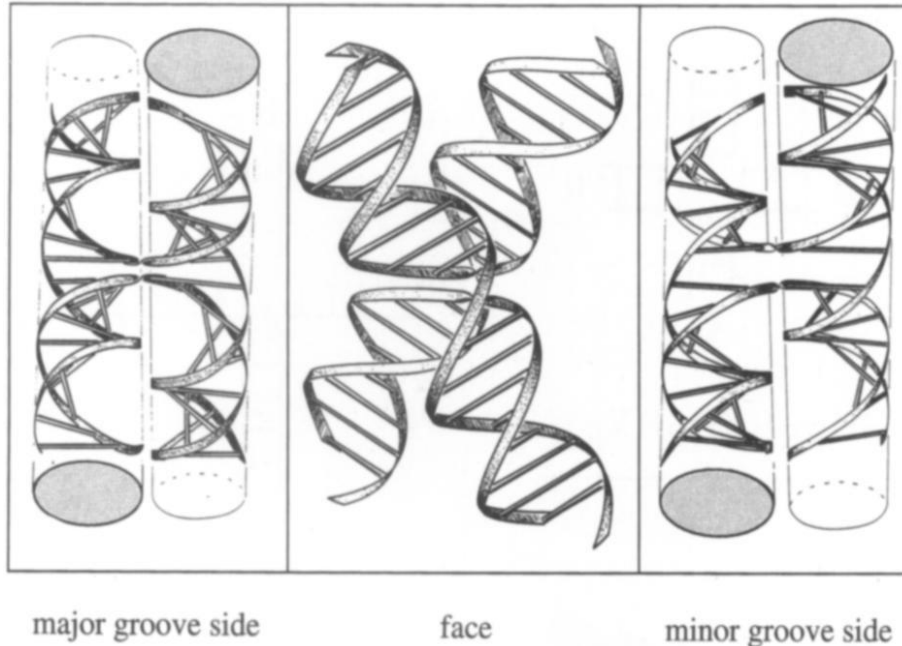
Figure 7: Ribbon model of the right-handed, antiparallel stacked X-structure of the four-way DNA junction, observed from three points of view. Note the juxtaposition of the continuous strands in the major grooves of the opposing helices, which is optimal for a small angle of 600 for the helix crossing. Centre: face view, showing the X-shape of the folded junction: the two sides of the structure are not equivalent. On one side (left) the four basepairs at the point of strand exchange all present major groove edges, while on the other side (right) the minor groove edges are presented.[2]

Immobilized 4WJs (Figure 2) were first synthesized by Seeman et. al. in 1983 using an algorithm to generate nucleic acid sequences that form junctions with high fidelity and stability.[18] The smallest reported 4WJ, which we label J1, has optimally high fidelity and thermal stability. With respect to stabilizing or "locking" 4WJ structures, Brogden et. al reported the formation of stabilized junction upon addition of a small molecule synthesized *in vivo*[19]. The prototypical compound was derived from a class of compounds called acridines. The molecule was designed to crossover the junction region and "bridge" the two DNA strands together. Electrophoretic studies showed that the DNA junction was greatly stabilized when bound to this molecule. Electron density maps of the resulting junction showed a marked increase in the crossover site of the

junction, which implies a stronger binding between the molecules present at that region. Another study by Howell and colleagues reported a similar experiment with another acridine derivative that behaved analogously to divalent cations such as magnesium and calcium to bind DNA junction and stabilizing it[20]. The acridine binds in an intercalative fashion to the DNA junction. They also found that a high temperature annealing step is still necessary even in the presence of high concentrations of cations to form the junction *in vitro*.

Peptide nucleic acids (PNA) and DNA/PNA hybrids

Peptide nucleic acid, or PNA, has a structure very similar to that of DNA, and in fact was designed as a DNA mimic that could recognize and bind to DNA[21,22]. The major difference is in the backbone of the two structures. DNA contains a phosphodiester backbone, PNA contains a pseudo-peptide backbone that also has the practical advantage of being neutral (Figure 8).

One interesting and useful characteristic of PNA-DNA interactions is that the binding between PNA/DNA complexes is much stronger than that of typical DNA/DNA structures. Since the PNA molecule is uncharged, there is no charge repulsion between charged DNA and neutral PNA[22] (Figure 6). Because of this feature, PNA/DNA complexes are more thermally stable, and more resistant to nucleases that (enzymes that cleave nucleic acids). One disadvantage to PNA, however, is its relatively lower solubility in water due to the neutral nature of of its peptide backbone.
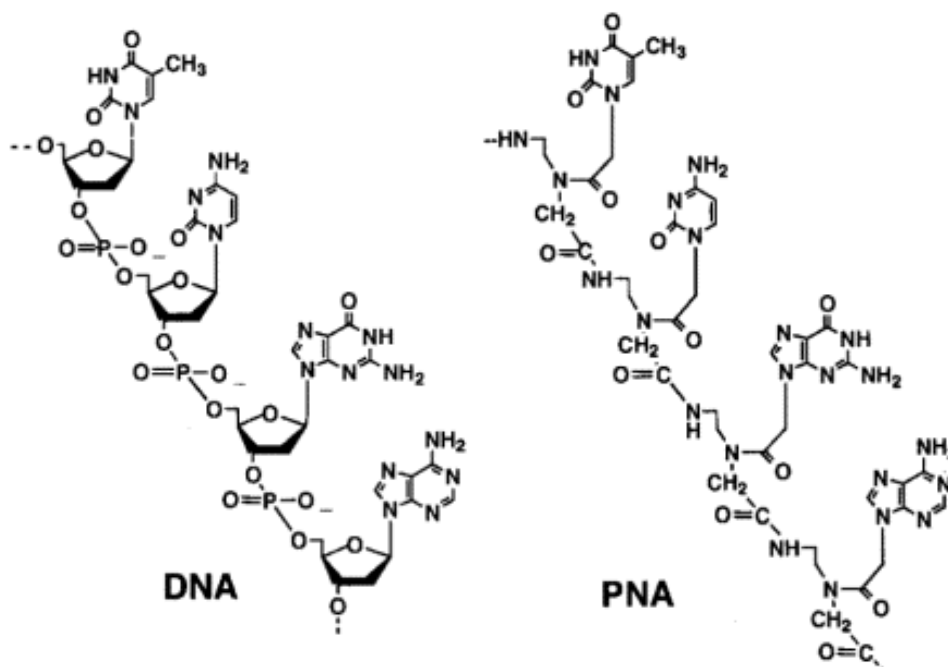
Figure 8: Comparison of DNA and PNA chemical structures. Shown here is a N-(2-amino-ethyl)-glycine backbone for PNA.[24]

A report by Musumeci and colleagues[8] describes the use of a PNA/DNA complex to bind HMGB1, with the aim of inhibiting cytokine activity. In the experiment, a double-stranded PNA/DNA chimera was used to bind HMGB1 *in vitro*, with positive results. The concentration of HMGB1 in chemotaxis assays decreased with application of the PNA/DNA chimera. However, the double-stranded complex used in the experiment has only one binding site for HMGB1, compared to four in a DNA junction. In addition, the binding site for the protein consisted of solely DNA, whereas in a PNA/DNA 4WJ the binding sites include PNA as well.

CHAPTER III – EXPERIMENTAL SECTION

Four-way DNA junction assay

The four oligonucleotides for J1 were purchased from Integrated DNA Technologies

(IDT). The sequences for the strands are shown below:

101:    5'-CGCAATCCTGAGCACG-3'

102:    5'-CGTGCTCACCGAATCGC-3'

103:    5'-GCATTCGGACTATGGC-3'

104:    5'-GCCATAGTGGATTGCG-3'

Oligonucleotide 101 was labeled with fluorescein. Fluorescein labeled strands (101 or

103) were purified via HPLC (IDT). Non-labeled strands were purified via denaturing

polyacrylamide gels (IDT). The four-way DNA junction was prepared by mixing the 101

labeled strand at 25 µM with the other three non-labeled strands at 5:1 excess (125 µM)

molar ratio. By making the 101 labeled strand the limiting factor we can therefore

assume that 100% of the detectable junction is formed at 25 µM.
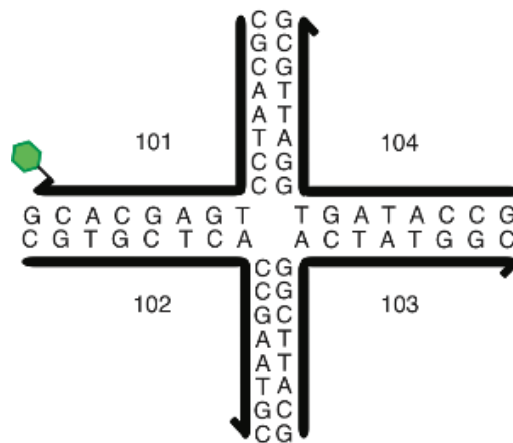
Figure 9: Schematic of the four-way DNA junction with
the 101 strand labeled with fluorescein at the 5' end.

The strands are then annealed at room temperature in 50 mM Tris-HCl (pH 7.5) and 10 mM MgCl$_2$, in the absence of light at variable times, to allow the single strands to base-pair with the complementary adjacent strands to form the junction.
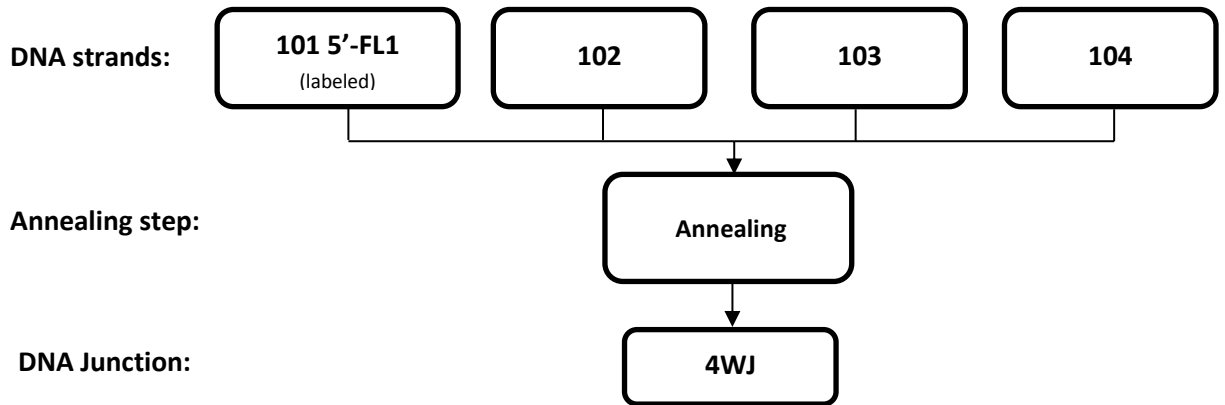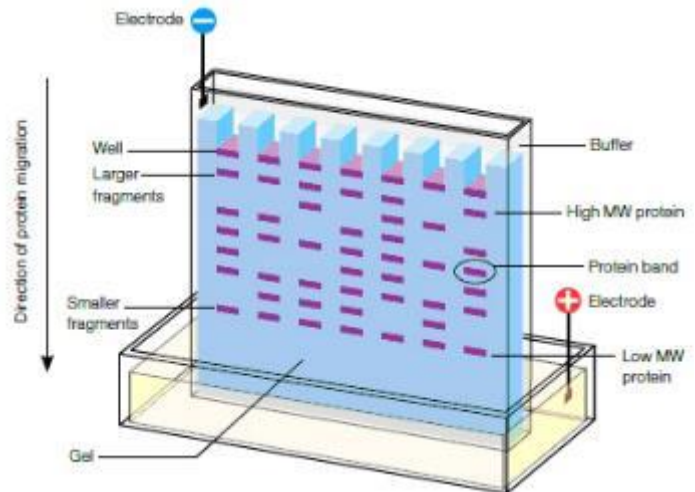

Figure 10: Steps for preparing 4WJ.

After the annealing step, 2 µL of each junction sample with compound are mixed with 6 µL nuclease free water, and incubated with 2 µL reaction buffer (125 mM Tris-HCl, 500 mM Nacl, 5 µM MgCl$_2$, 20% glycerol, pH 8.0) on ice for 30 minutes, before loading onto polyacrylamide gel electrophoresis (PAGE) gels. Electrophoretic mobility shift assays (EMSAs) are carried out with the resulting junctions using a Bio-Rad Mini-PROTEAN® Tetra Cell system with 15% polyacrylamide gel for 1 – 5 hours at 50 V in 4° C. The running buffer was 0.5 x TBE (45 mM Trisma, 45 mM boric acid, and 1.0 M EDTA), pH 8.0, with 1 µM MgCl$_2$ present in solution. After the gels are run, they are dried on Whatman 3MM paper and visualized using a Typhoon 9400 Phosphorimager. Labeled single strand is included in the assays as control.
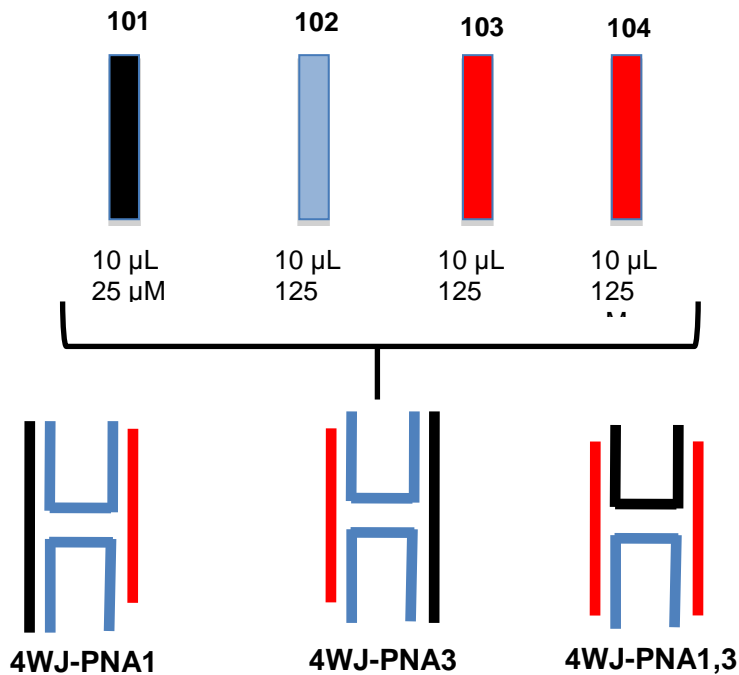
DNA-PNA hybrid junctions:

PNA strands were incorporated into the junction by replacing the DNA strand at the desired position with the complementary PNA strand. Different combinations of DNA-PNA hybrids can be achieved by modifying which PNA strand is incorporated into the mixture. 4WJ-PNA$_1$ was composed of PNA1: H-CAATCCTGAGCA-K-NH$_2$ and oligonucleotides 102, 103 and 104.   In this case, the labeled strand 103 was mixed with 5-fold excess of unlabelled PNA1, 102 and 104. 4WJ-PNA$_3$ was composed of PNA3: H-ATTCGGACTATG-K-NH$_2$, 101, 102 and 104. In this case, the labeled strand 101 was mixed with 5-fold excess of unlabelled 102, PNA3 and 104. The mobility of the hybrid 4WJ in the EMSAs was compared to that of the all-DNA 4WJ, as well as a labeled 101 single strand.

To perform EMSAs with 4WJ in the presence of small molecules, the small molecules are prepared by dissolving the crystals in water at various concentrations. Dilutions are prepared in nuclease-free water or annealing buffer containing MgCl$_2$. The small molecules are then added to the 4WJ mixture before the annealing step. The resulting mixture is run on PAGE gels under the same conditions as before.

Figure 12: Preparation of DNA-PNA hybrids. The red strands are PNA strands, and the black is labeled DNA, and the blue is unlabeled DNA. Shown here is the stacked-x form of the 4WJ.

Junction assays in the presence of small molecules:

A series of compounds available for purchase from Sigma-Aldrich™ were tested for their binding capacity with 4WJ. The compounds were chosen for their commercial availability, known DNA binding motifs (polycyclic, aromatic, and planar), and the reported solubilities in water (based on K_{sp} values).

Table 1: Initial set of compounds obtained from Sigma-Aldrich™:

	Compound Name	Molecular Structure
Compound A	3-[[(9H-Fluoren-9-ylmethoxy)carbonyl]amino]-2,3,4,5-tetrahydro-2-oxo-1H-1-benzazepine-1-acetic acid	
Compound B	3-([(9H-fluoren-9-ylmethoxy)carbonyl]amino)-5-hydroxybenzoic acid	
Compound C	4-Carbamoyl-2-([(9H-fluoren-9-ylmethoxy)carbonyl]amino)butanoic acid	
Compound D	N,N'-Bis-trityl-hexane-1,6-diamine	

In spite of the reported K_{sp} values, the compounds were very difficult to dissolve in Tris

buffer or water at room temperature, so the above set of compounds were not used for

further experiments.

Another molecule was obtained from Dr. Alvin Holder at The University of

Southern Mississippi. The molecule, [Ru(bpy)$_2$(dpp)PtCl$_2$]Cl$_2$, or Ru(bpy)$_2$ (Figure 6) is

used because of its structural similarities with other known DNA binding substrates, and

its high solubility in water.
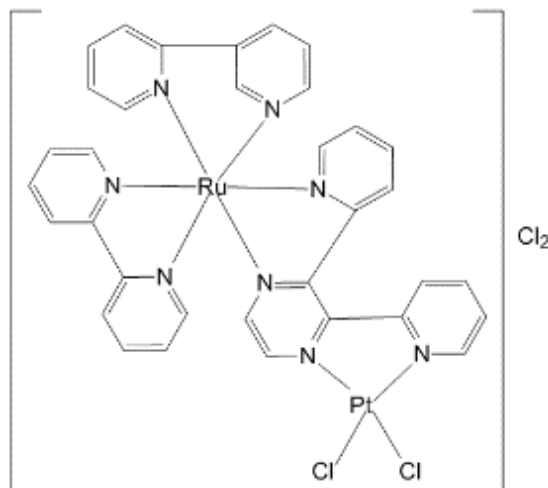
Figure 13: Structure of the Ru(byp)$_2$ molecule.

UV-Vis analysis of [Ru(bpy)$_2$(dpp)PtCl$_2$]Cl$_2$:

The Ru(bpy)$_2$ compound is characterized via

ultraviolet and visible wavelength emission

spectroscopy (UV-Vis) to find absorption

peaks. For UV-Vis, the Ru(bpy)$_2$ samples are

The Beer-Lambert Law:

$A = \varepsilon \times l \times c$

Where A is absorbance,
            ε is molar absorptivity in L mol^{-1} cm^{-1}
            l is the path length, in cm, of the cuvette
              containing the sample
            c is the concentration of the compound
              in solution, in mol L^{-1}

prepared in annealing buffer and/or water, at varying concentrations in the nanomolar

range. The samples are then placed in a cuvette with a path length of 1 cm. Scans were

run for a wavelength range of 200 nm to 600 nm on a GE Ultrospec 8000. Using the

Beer-Lambert Law the maximum absorbance peaks of Ru(byp)$_2$ can be determined.

Once the absorbance peaks of Ru(byp)$_2$ are known, they can be compared against

substrates of the molecule – in this case DNA junction – to observe binding between the

4WJ and Ru(byp)$_2$. To incorporate the Ru(byp)$_2$ molecule into the 4WJ mixture, the molecule is dissolved in nuclease-free water and/or annealing buffer, and added before the annealing step.

HMGB1 expression and purification:

HMGB1b and HMGB1b/R26A from rat were expressed from pHB1-*Escherichia coli* Bl21(DE3)pLysS. Each protein was purified via FPLC using an Econo-Pac CM cartridge (Bio-Rad). Crude proteins were loaded onto the CM cartridge in the presence of low salt buffer: 50 mM Tris-HCl (pH 7.0), 50 mM NaCl and eluted with high salt buffer 50 mM Tris-HCl (pH 7.0), 500 mM NaCl using a linear gradient. Human histone H1 was purchased from New England Biolabs. The purity of each protein was monitored by resolution of each sample on 12% SDS-polyacrylamide (29:1 acrylamide:bisacrylamide) gels in Tris-Tricine buffer (150 V for 45 minutes) followed by staining for 12 hours with Coomassie Brilliant Blue G-250.

# CHAPTER IV – RESULTS AND DISCUSSION

Electrophoretic mobility shift assays (EMSA) of J1 and PNA-DNA hybrid junctions:

All gels were scanned using a Typhoon 9400 Phosphorimager.
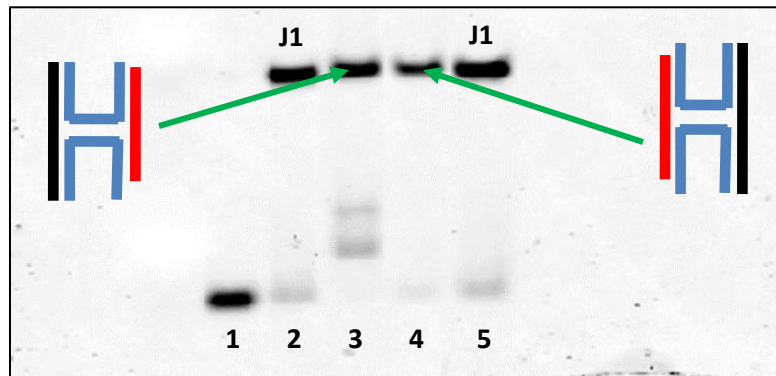
Figure 14: 15% Native gel of 50 nM DNA and hybrid PNA-DNA 4WJs. Lane 1: 101 single-strand control, lane 2 and 5: J1, lane 3: 4WJ-PNA$_1$, lane 4: 4WJ-PNA$_3$.

Figure 14 shows an EMSA of the all-DNA junction J1 loaded next to two PNA-DNA hybrids: 4WJ-PNA$_1$, and 4WJ-PNA$_3$. The dark bands show the mobility of the junctions down the gel. Lane 1 contains a labeled 101 single-strand control that has a high mobility compared to the junctions due to its relatively smaller size. It is important to note that the all-DNA J1 and the two PNA-DNA hybrids have the same mobility through the native gel. This gel data allows us to presume that DNA 4WJ and hybrid 4WJ can thus be examined using EMSAs and their binding properties can be compared with one another.
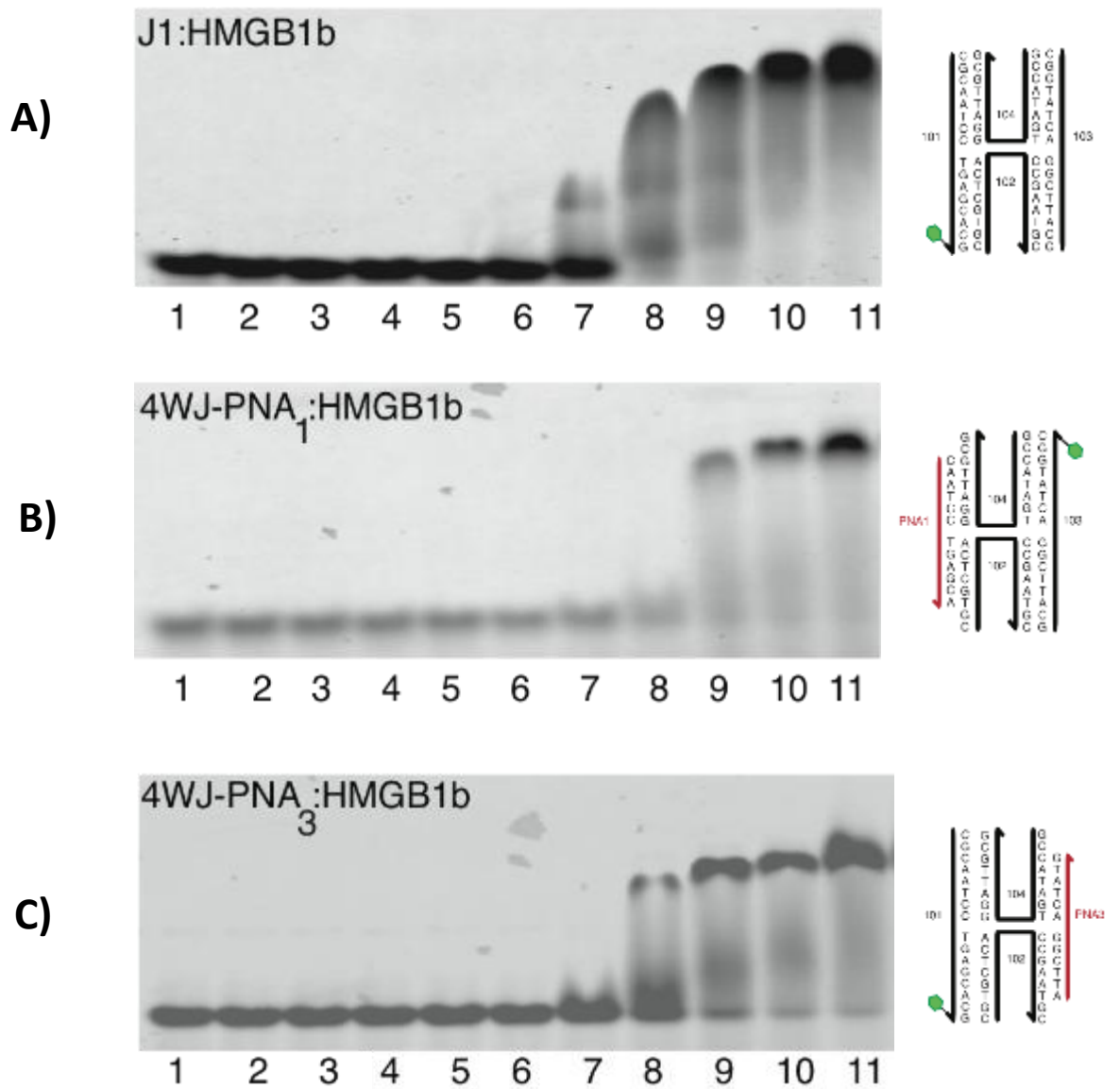
Figure 15: Native EMSAs of 4WJs and HMGB1b. Lane 1: 50 nM 4WJ, lanes 2 – 11; J1 + HMGB1b at protein : junction molar ratios of: 1:1, 2:1, 4:1, 8:1, 16:1, 32:1, 64:1, 128:1, 256:1, and 478:1.

Figure 15 shows EMSAs of J1 and two PNA-DNA hybrids binding with increasing

concentrations of HMGB1 b-box. Figure 15A shows J1 in the presence of HMGB1b, 15B

shows the 4WJ-PNA$_1$ hybrid with HMGB1b, and 15C shows 4WJ-PNA$_3$ and HMGB1b. At

protein : junction molar ratios of 128:1, 256:1, and 478:1, the hybrid junctions show

high binding affinities to HMGB1b that are comparable to the all DNA J1. For 4WJ-PNA$_3$,

the bands at 32:1 and 64:1 molar ratios appear to be stronger than that of J1, suggesting

a slightly better binding to the protein.

Once the stability of the PNA-DNA hybrid junctions was established, the

junctions were tested for thermostability. EMSAs were performed by annealing the

junctions at elevated temperatures (37°C and 57°C).
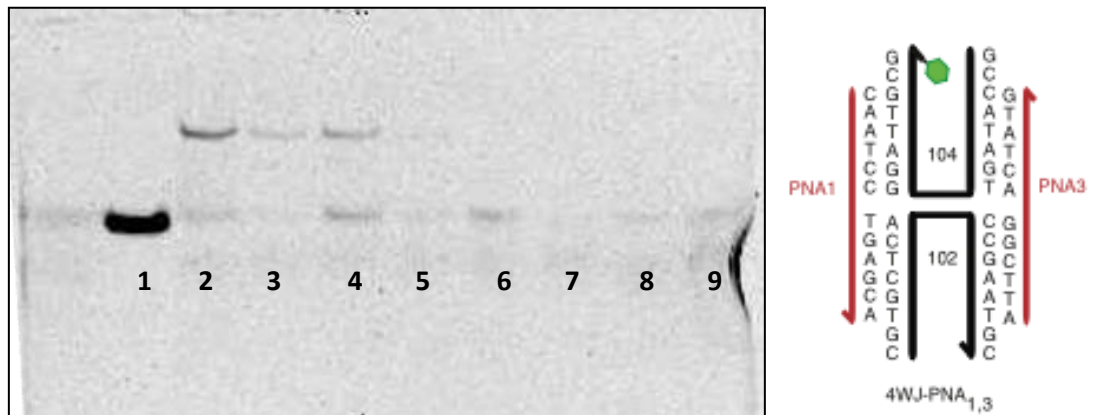
Figure 16: Native gel of 50 nM 4WJ-PNA$_{1,3}$, 15% PAGE gel run with 0.5X TBE buffer at 4° C for 90 minutes.
1) 101 ss control
2) 4WJ-PNA$_{1,3}$ – 4°C 10 mM MgCl2
3) 4WJ-PNA$_{1,3}$ – 4°C 50 mM NaCl
4) 4WJ-PNA$_{1,3}$ – RT 10 mM MgCl2
5) 4WJ-PNA$_{1,3}$ – RT 50 mM NaCl
6) 4WJ-PNA$_{1,3}$ – 37°C 10 mM MgCl2
7) 4WJ-PNA$_{1,3}$ – 37°C 50 mM NaCl
8) 4WJ-PNA$_{1,3}$ – 57°C 10 mM MgCl2
9) 4WJ-PNA$_{1,3}$ – 57°C 50 mM NaCl

Figure 16 shows an assay with 4WJ-PNA$_{1,3}$ after annealing at 37°C and 57°C. The

junctions do not maintain their integrity at elevated temperatures. Further studies are

required with other combinations of PNA-DNA hybrids, as well as three-way DNA

junctions.
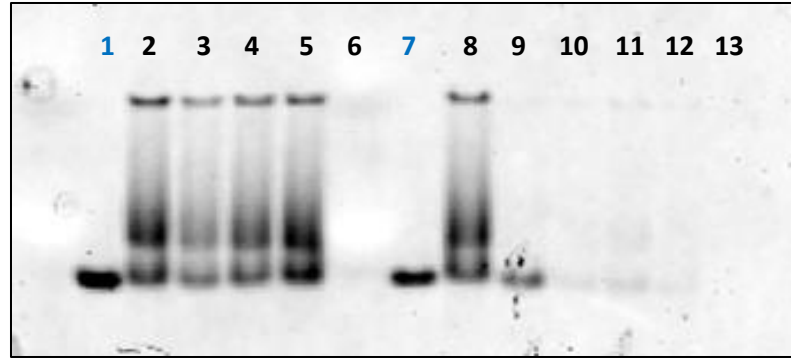
Figure 17: Native gel of J1 samples at 50 nM, run at 4° C (2 hrs) on a 15% native gel. Lanes 1 and 7 are nucleic acid markers, lanes 2 and 8 are the single strand control. Lanes 3 - 6 and 9 contained J1 samples annealed via SpeedVac with increasing concentrations of $Ru(bpy)_2$ added, and lanes 10 - 13 contain J1 that had been annealed at room temperature in the presence of increasing concentrations of $Ru(bpy)_2$: 1.0 nM, 10 nM, and 100 nM.

Figure 17 shows an EMSA with J1, the all-DNA junction, annealed in the presence of $Ru(bpy)_2$. Lanes 3 – 6 contain J1 that has been prepared via SpeedVac and lanes 10 – 13 contain J1 that was annealed at room temperature, both in the presence of increasing concentrations of $Ru(bpy)_2$. The dark bands at the top of the gel are due to junction formation. The gel shows that even at nanomolar concentrations of $Ru(bpy)_2$, the junction formation is comparable to J1 without any $Ru(bpy)_2$ present. However, J1 annealed at room temperature was not efficient and could not be elucidated very well, although some junction formation is still evident (very light bands at the top).
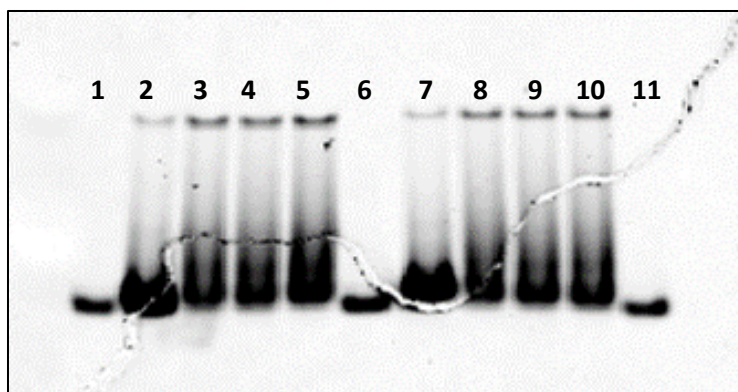
Figure 18: Native gel of J1 at 500 nM, same conditions as before. Lanes 1, 6, and 11 are the 101 ss controls; lanes 2 and 7 are J1 without Ru(byp)$_2$; and lanes 3 - 5 and 8 - 10 are J1 with 1.0 nM, 10 nM, and 100 nM Ru(byp)$_2$.

To improve detection of the sample, the assay was repeated for the samples at room temperature, but with a higher concentration of J1. Figure 11 shows J1 at 10x higher concentration than the one in Figure 10. As a result, the bands can be much better elucidated. The dark bands suggest that J1 forms in the presence of Ru(byp)$_2$ at 1.0 nM, 10 nM, and 100 nM concentrations(lanes 3 – 5 and 8 – 10) , and with relatively better efficiency than just the J1 without Ru(byp)$_2$ (lanes 2 and 7).

CHAPTER V – CONCLUSION

Our data shows that a Ru(byp)$_2$ can effectively assist the assembly of native and hybrid junctions in the presence of MgCl$_2$. This is a significant achievement because one of the long-term goals of this project is to use DNA junctions to target the proinflammatory cytokine HMGB1. Prior research[10] has clearly shown that hybrid 4WJs can be constructed and these molecules bind to HMGB1b and the DNA biding protein Histone H1 with high affinity. More recently, research has shown that the hyrbrid 4WJ, 4WJ-PNA$_{1,3}$ posseses enhanced resistance to endo- and exonucleases. Moreover, the hybrids 4WJ-PNA$_1$ and 4WJ-PNA$_3$ possess enhanced stability in fetal bovine serum (FBS) vs. J1. The *in vitro* and serum stablity data are very encouraging but the thermostabilty all 4WJs tested do not exceed 42°C (data not shown). We presume that enhanced levels of thermostabilty will required for the use of these molecules as therapeutics. Our initial investigations show that Ru(bpy)$_2$ is capable of forming stable 4WJ and three-way (3WJs) at room temperature. This data correlate with those from Howell et al.[20] A similar approach was used by Grueso et. al.[23], in which they used a phenanthroline-derived ruthenium complex to bind to DNA via intercalation. Their data showed that Ru(byp)$_2$ enhanced the thermostabilty of calf thymus DNA by > 15°C. Based on this, we presume that Ru(byp)$_2$ may enhance the stability of native and hybrid 4WJs. Preliminary EMSA data indicates that Ru(bpy)$_2$ does not significantly enhance the stability of the hybrid 4WJ-PNA$_{1,3}$ (Figure 16). The EMSA data is very informative but these assays do not provide an accurate measurement of the thermostability of nucleic acids. Hence, circular dichroism (CD) and UV spectroscopy will be used for future investigations of thermostability.

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