

DNA i-motif Stabilization by Ligand Binding to Loop Regions

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Abstract

DNA i-motifs are a secondary structure of DNA that can be useful in a variety of ways, but this experiment investigates the maximum potential to stabilize i-motifs artificially with a small molecule binding to loop regions. DNA with different position and number of loops was tested to see to what extent the i-motif could be stabilized. A drug, Actinomycin D, was used for its known high affinity for a particular DNA sequence, which we used to make the loops of the i-motif. The binding constant for the drug to the DNA was determined through fluorescence spectroscopy while the presence of the i-motif structure was confirmed using Circular Dichroism spectra. The resulting dissociation constants showed that AMD had a higher affinity for the DNA strands when in the i-motif conformation at pH 5.4. The small molecule had the greatest binding constant when more than one binding site was present except in the case of L13HP due to steric hindrance by the nature of the i-motif structure.

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Introduction:

While DNA is usually thought of in its common duplex formation, it is capable of forming many different secondary structures. Two of the secondary structures that single-stranded DNA (ssDNA) are capable of forming are G-quadruplexes (G4s) and i-motifs (iMs). Whether these conformations are present *in vivo* has been an area of particular interest in recent years. Balasubramanian et al., demonstrated the existence of G quadruplexes *in vivo* in 2013 (1), but iMs were thought to not be physiologically relevant because they are only stable at pHs much more acidic than that present in the nucleus of cells. The pH dependence is due to the necessity of a hemiprotonated cytosine—cytosine base pair (2), as shown in fig. 1.

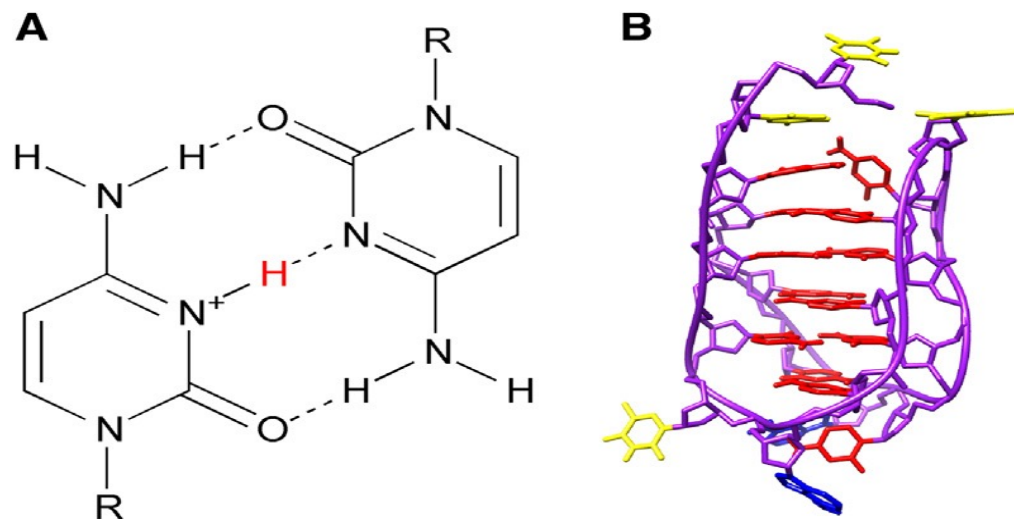


Figure 1: C-rich DNA structure of iM. (A) Hemiprotonated C—C⁺ bond, which is the basis for iM stability. The shared proton is highlighted in red. (B) An all-atom molecular model of an iM modified from PDB 1YBL (10). The bases are color coded for thymine (yellow), cytidine (red), adenine (blue), and the DNA phosphate backbone (purple)(11).

However, iMs have recently been shown to form at cytosolic pH when molecular crowding agents that mimic the congested environment of cells are present because they shift the pK_a of iMs to physiologically relevant pHs (3). Thus, iMs are now thought to have the potential to be viable *in vivo*.

G quadruplexes consist of guanine rich DNA and iMs C rich DNA. They both have been thought to form in parts of DNA that regulate cell growth (1). This is important because the presence or absence of secondary structures may act as switches to control gene expression (4). This is not a new idea as the same theory has been applied to ssDNA that are capable of forming a hairpin or cruciform. These secondary structures exist in equilibrium and often undergo interconversion between folded and unfolded structures. To manipulate this equilibrium, small molecules or transcription factors can be used to bind and stabilize a desired structure (5). In theory, a small molecule could stabilize a secondary structure while in the “off” position to prevent transcription and thus expression of a gene.

Actinomycin D (AMD) is a small peptide-containing natural product drug that has been used previously to treat tumors. This drug is thought to inhibit transcription through the binding of DNA and blocking RNA polymerase during elongation, thereby preventing transcription and hence cell proliferation (6). Later the drug was found to bind with high affinity and sequence specificity to ssDNA, which provided an additional model for the

method of transcriptional inhibition where ssDNA is bound in the open complex formed by the polymerase, preventing the ssDNA strands from reannealing. Furthermore, structures formed by ssDNA such as hairpins and cruciform DNA are high affinity sites for AMD due to the pocket formed by the loop region of the ssDNA (7).

The idea of binding ligands to iMs to stabilize the structure is not a new one. Previous work has been done to try to find a ligand that will target iMs and potentially lead to novel drug design (8). Experiments were conducted with TMPyP4, and while the K_d was determined to be $\sim 1\mu\text{M}$, the ligand is able to bind to many different DNA structures, which creates a problem for targeting iMs specifically. One of the iM ligands that has been studied the most are carboxyl-modified single-walled carbon nanotubes (SWNTs). SWNTs have specificity for iMs, but further study showed that DNA damage, displacement of telomere-binding proteins, and telomere uncapping occurred as a result of treatment with SWNTs. Relatively few iM specific ligands have been identified, and there is a need for further structural understanding of iM binding, as well as understanding of the dynamics and conformation of ligand binding.

Due to its high binding affinity for DNA loops with selected sequences ($K_d \sim 1\mu\text{M}$) AMD was used in this study to determine how effective a small molecule could be in stabilizing iMs. Because AMD has such a great affinity for the sequence AGTTTAAA located in a potential loop, many different ssDNAs with one, two, or three loops containing his sequence were

examined. The experiments described in this thesis were conducted to determine the relative affinity of AMD for DNA loops in unfolded ssDNA at pH 8.0 and when the DNA was folded into an i-motif at pH 5.4. Our hypothesis is that when AMD is bound to the loops of iM DNA, it will confer enhanced thermodynamic stability to the iM and thus may form the basis for the design of small molecule drugs to target potential iMs *in vivo*.

Materials and Methods:

All DNA oligos were synthesized through standard solid-phase chemistry by Midland Certified Reagent Co., Inc. (Midland, TX). The oligos were stored in 20 mM NaCl, 10 mM Tris, 0.1 mM EDTA buffer (pH 8.0) until they were used.

7-aminoactinomycin D (7AAMD) was used because of its fluorescence properties (9). Specific DNA sequences based on the simple T1 sequence 5'-CCCTCCCTCCCTCCC-3' capable of forming iMs that also contained specific AMD-binding loops were examined. The single stranded DNA sequences used in this report are given below, with the AMD-binding loop sequences shown in red:

L1HP: 5'-CCCAGTTTTAAATCCCTCCCTCCC-3'

L2HP: 5'-CCCTCCCAGTTTTAAATCCCTCCC-3'

L3HP: 5'-CCCTCCCTCCCAGTTTTAAATCCC-3'

L12HP: 5'-CCCAGTTTTAAATCCCAGTTTTAAATCCCTCCC-3'

L23HP: 5'-CCCTCCCAGTTTTAAATCCCAGTTTTAAATCCC-3'

L13HP: 5'-CCCAGTTTTAAATCCCTCCCAGTTTTAAATCCC-3'

L123HP: 5'-CCCAGTTTTAAATCCCAGTTTTAAATCCCAGTTTTAAATCCC-3'

Titration curves

The binding of 7AAMD to the DNAs was determined using fluorescence spectroscopy. Fluorescence was excited at 530 nm and emission intensity was acquired from 560-700 nm. The titration buffer used was 20 mM sodium cacodylate for pH 5.4, and 20 mM Tris for pH 8.0. The 7AAMD was added to the buffer solution to reach a concentration of 0.6 μ M. Then, 1 mM of ssDNA was titrated into the drug solution in increments of 0.5 – 3.0 μ L, until saturation was apparent. Fluorescence emission spectra were recorded after each addition of DNA.

Circular Dichroism

An Olis DSM 20 circular dichroism (CD) instrument fitted with Peltier heat block (Olis, Inc., Bogart, Ga) was used to collect CD spectra. Data were collected from 350-220 nm. An integration time as a function of high volts was used. A baseline from 750 microliters of buffer was also used. To make a 5 micromolar solution, 3.75 microliters of each particular strand of DNA were added to the buffer solution. Data was gathered for each DNA strand at both pH 8.0 and 5.4.

After K_d values were determined, to confirm the binding of 7AAMD to the ssDNA in the iM formation, 15 microliters of 7AAMD was added to the 5 micromolar solution of ssDNA at pH 5.4 to ensure >90% of ssDNA would have 7AAMD bound to it using a standard saturation curve for reversible binding created by the formula:

$$M_L = \frac{(L_0 + M_0 + K_d) - ((L_0 + M_0 + K_d)^2 - 4M_0L_0)^{1/2}}{2}$$

Where M_0 is the free macromolecule (the ssDNA), L_0 is free ligand (7AAMD), and M_L is the macromolecule-ligand complex. This formula has an underlying assumption that there is one binding site. This was also conducted at pH 8.0 with each DNA strand for comparison.

Results:

Table 1: K_d values for 7AAMD binding to ssDNA strands

	L1HP	L2HP	L3HP	L12HP	L13HP	L23HP	L123HP
pH 8.0	2.68	1.99	2.78	0.99	2.21	0.74	0.49
pH 5.4	1.58	1.11	0.42	1.08	1.67	0.49	0.44

Fluorescence spectra were gathered and used to determine the K_d for each DNA strand at pH 8.0 and 5.4 (Table 1). L1HP, L2HP, L3HP, L13HP, L23HP, and L123HP had a lower K_d at pH 5.4 when the DNA was in the iM formation. While L12HP did not have a decrease in the K_d values from pH 8.0 to 5.4 had only a very slight increase the dissociation value. All K_d values containing a loop in the 3 position at pH 5.4 were lower than other ssDNA without a loop in the 3 position except for L13HP. The ssDNA with two loops or more all had significantly lower K_d values except for L13HP. L3HP and L13HP at pH 8.0 were significantly less favorable targets for AMD, while all strands with a loop in the 2 position had the most favorable binding constants at pH 8.0.

CD spectra were gathered and used to identify the conformation of the ssDNA with AMD (Figure 6) and without AMD (figure 5) at pH 8.0 and 5.4. Evident by the horizontal shift of the peak to the left and the greater magnitude of the peak, the spectra confirmed that the ssDNA was in the iM conformation at pH 5.4 with and without AMD present. While the CD spectra does have a higher peak and horizontal shift for the ssDNA at 5.4, the CD

spectra at pH 8.0 has a smaller peak and does not have the characteristic shift of the iM, but the spectra is not characteristic of solely unfolded DNA.

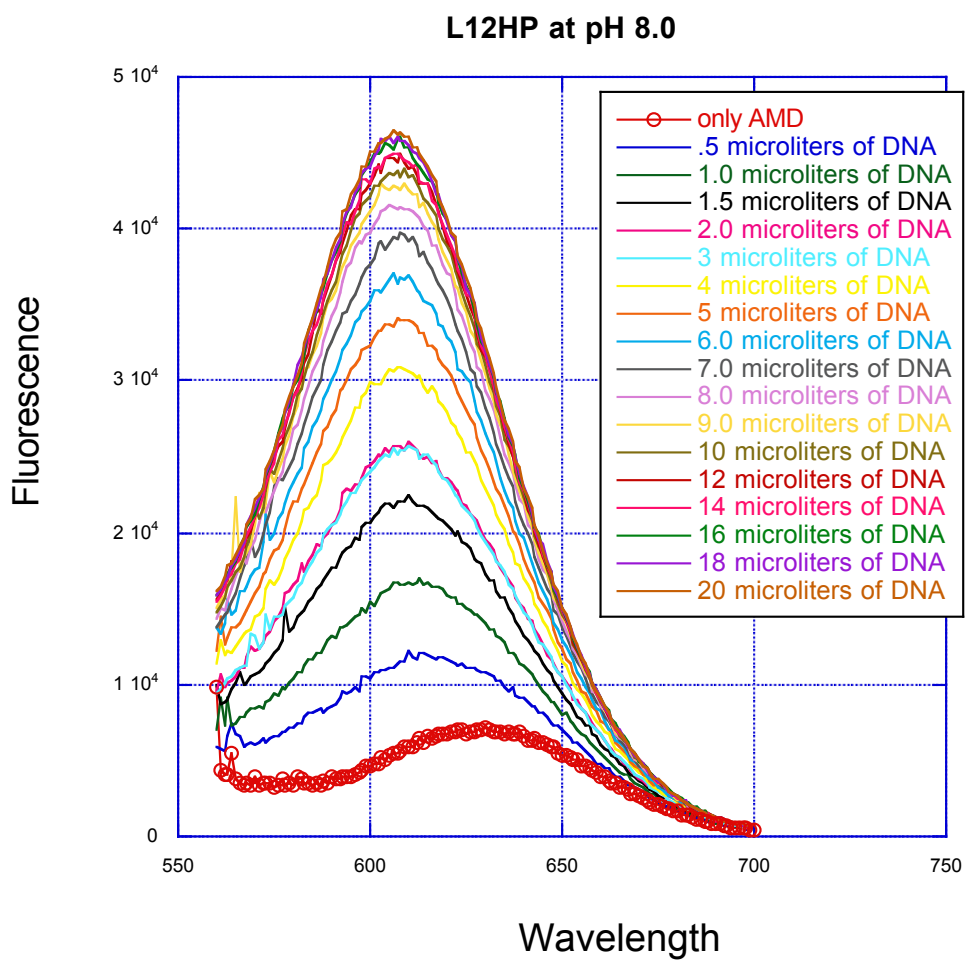


Figure 2: Fluorescence spectra of 7AAMD titrated with the ssDNA with loops in the 1 and 2 position (L12HP) at a pH of 8.0. Fluorescence was measured after each addition of DNA in the 0.6 μ M 7AAMD solution to determine the K_d .

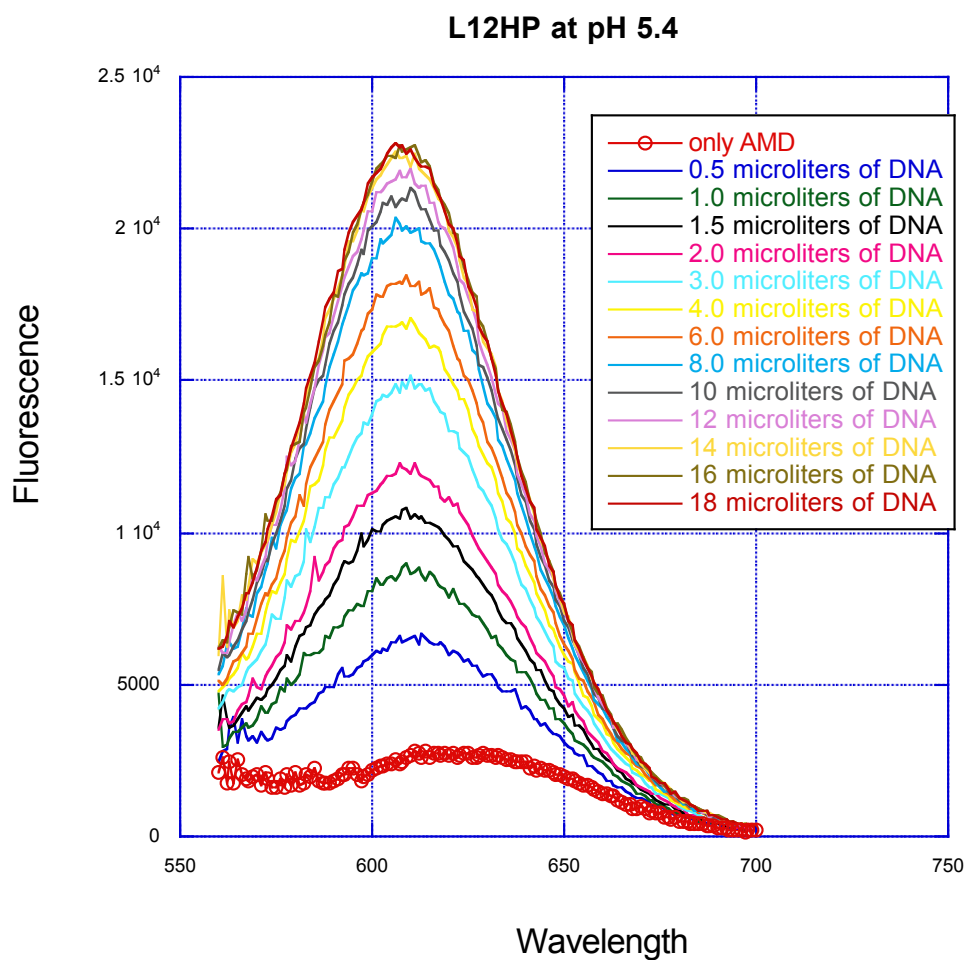


Figure 3: Fluorescence spectra of 7AAMD titrated with the ssDNA with loops in the 1 and 2 position (L12HP) at a pH of 5.4. Fluorescence was measured after each addition of DNA in the 0.6 μ M 7AAMD solution to determine the K_d .

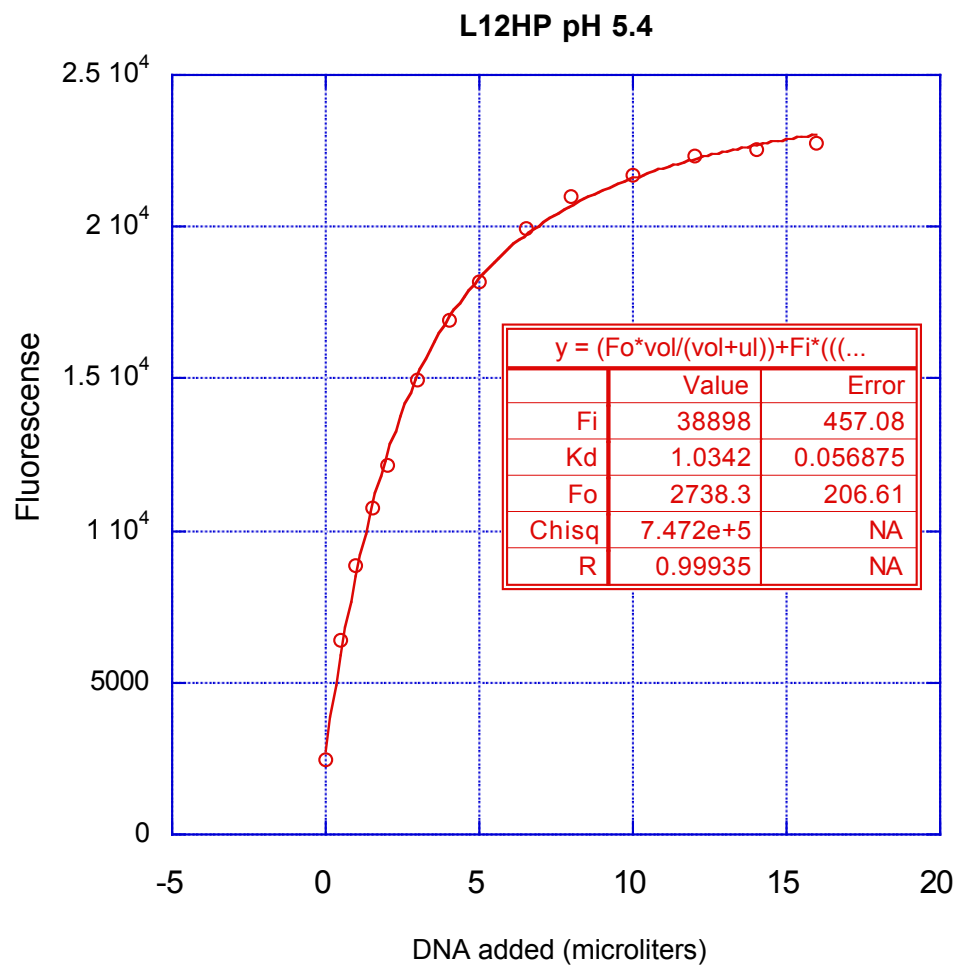


Figure 4: Hyperbolic curve for 7AAMD binding to ssDNA L12HP at pH 5.4 created from fluorescence spectra to produce a curve fit to calculate the K_d value.

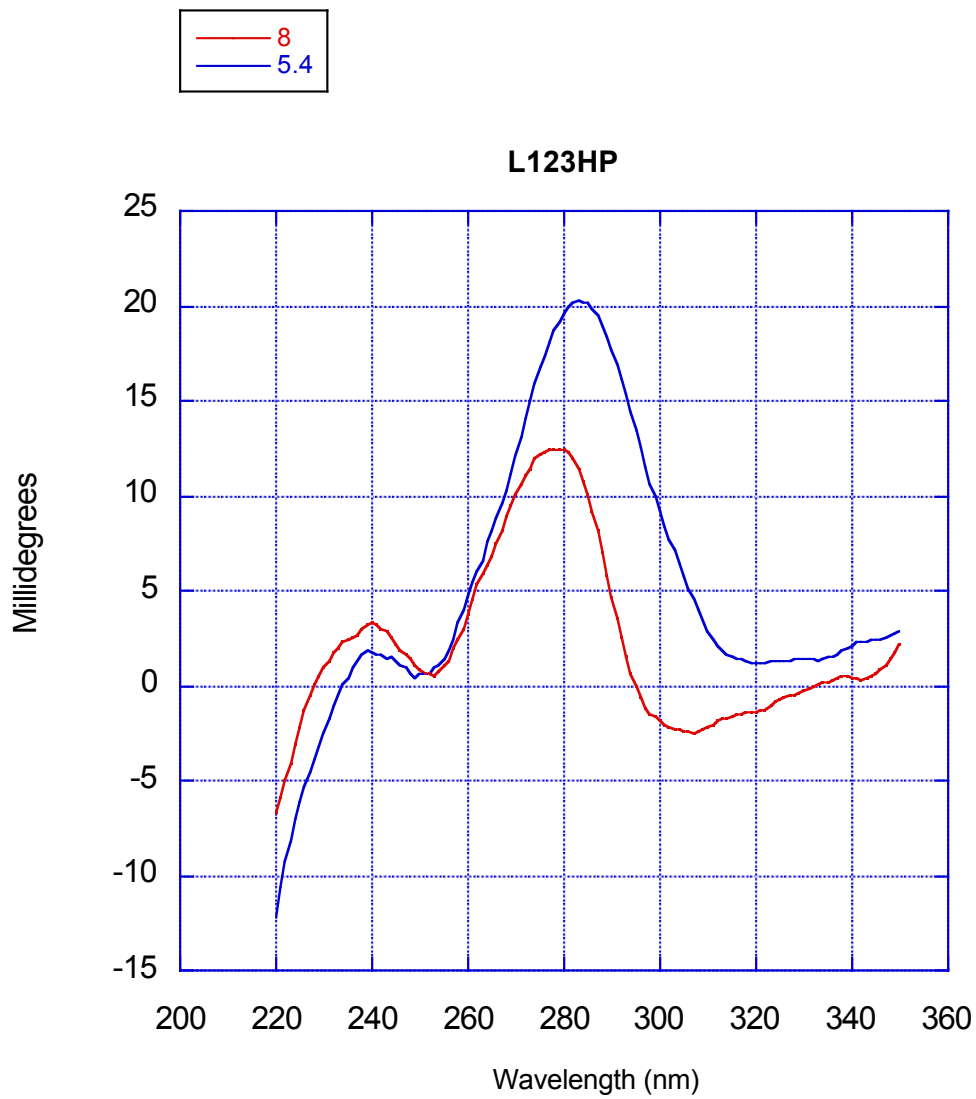


Figure 5: CD spectra at pH 8.0 (red) and 5.4 (blue) for ssDNA with with loops in positions 1,2, and 3 without 7AAMD. Horizontal shift and greater magnitude at pH 5.4 confirm the ssDNA is in the iM formation

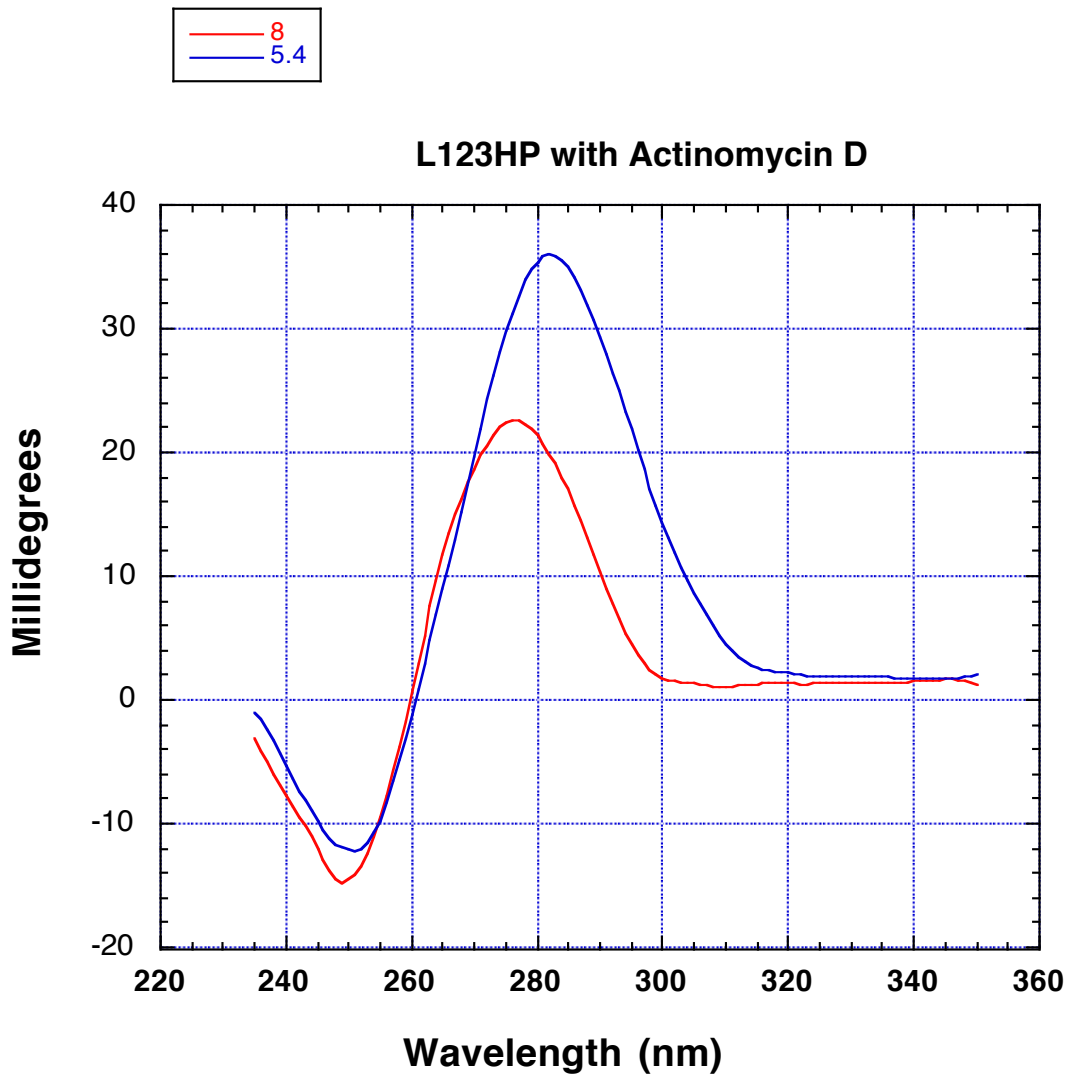


Figure 6: CD spectra at pH 8.0 (red) and 5.4 (blue) for ssDNA with with loops in positions 1,2, and 3 with 7AAMD. Horizontal shift and greater magnitude at pH 5.4 confirm the ssDNA is in the iM formation when 7AAMD is bound to it at pH 5.4.

Discussion:

The K_d values were significantly lower for most of the DNA strands at pH 5.4 because of the loop or loops created by the iM conformation that create a pocket for the pentapeptide ring in AMD. The L13HP strand was slightly unfavorable for the binding of AMD because of the position of the loops. In the iM formation, loops 1 and 3 are next to each other and thus steric hindrance makes it difficult for AMD to bind to both, while loop 2 is free and in the open due to the structure of iMs. The ssDNA strands that contain more than 1 loop generally had a lower K_d value because the small molecule had multiple binding sites to bind and stabilize the structure.

Some of the data obtained was very noisy due to an oxidative product formed in the AMD solution. With more work, data could be gathered to verify the work from when the small molecule had an oxidative product. One-way analysis and means comparison determined which data was significantly different and would need to be verified.

While to this point, the information has been gathered to determine the affinity of AMD for ssDNA in the iM conformation, more work is necessary to gain insight into the degree of stability of iMs that can be accomplished with a small molecule. Thermal melting curves for each ssDNA with the conditions necessary to have 90% of DNA with AMD bound would determine the thermal stability that iMs could have with a small molecule binding to the loop region. To determine the pK_a stability of iMs with small

molecules binding to the loop regions, experiments would be run on the CD at the conditions necessary to have 90% of DNA with AMD bound. Further experiments could also provide insight to the nature of the binding of 7AAMD to ssDNA at pH 8.0. If the drug confers stability to DNA at pH 8.0 in the iM conformation, it would mean the drug has the ability to shift the pK_a of the iM similarly to molecular crowding agents.

One-way Analysis of K_d values By DNA

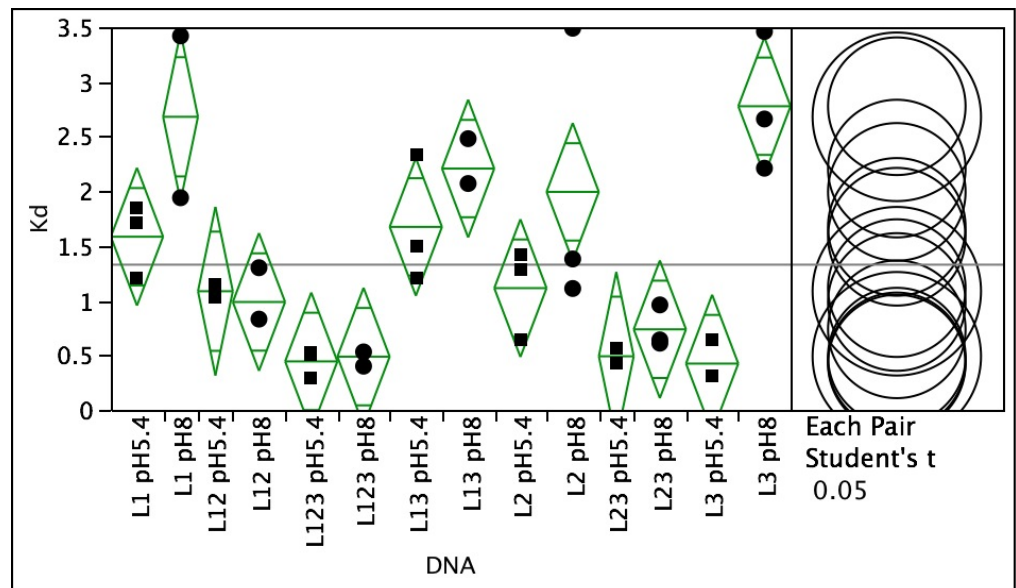


Figure 7: One-way analysis was performed to compare the numerical data that was gathered to determine what data had significant differences

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