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Determination of Intercalating Behavior of Bis-cation Imidazolium Salt Systems

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Abstract

Many chemotherapeutic drugs have had success in treating a variety of cancers, but some can be harmful and cause adverse side affects such as hair loss and nausea. Cisplatin is a well-known chemotherapy drug that has been used to treat a countless number of different types of cancers including non-small cell lung cancers (NSCLC). Because of its nonspecificity and secondary effects, investigation into alternative non-small cell lung cancer targeting drugs is necessary. The recently synthesized bis-cation imidazolium salt system has displayed antiproliferative activity, but its mechanism of action remains undetermined. The system explores the use of two napthalenes, two imidazole salts, and linking carbon chains varying from 1-12 carbons in length. Here, the system was tested for possible DNA intercalation using fluorescent intercalator displacement (FID) and viscosity experimentation. Results from FID exhibit negligible displacement activity of ethidium bromide from the 4 selected compounds. In contrast, viscosity results demonstrate 3 of 4 compounds showing greater DNA interaction compared to the acridine orange control. These opposing conclusions ultimately determine DNA interaction is present, but not strong enough to effectively displace a known intercalator. Overall, results do not concretely reveal a mechanism of DNA interaction.

Introduction

Of the 224,390 predicted new cases of lung cancer in 2016 over 83% will be non-small cell lung cancers (NSCLC).[1] Treatments for lung cancer vary from surgery, radiation, to chemotherapy and the method and drug chosen for treatment are specifically tailored to each patient. Cisplatin proves to be very effective and widely used as a chemotherapy drug, but it is known to cause harmful side effects to non-cancer cells.[2] Improving upon current cancer-targeting drugs leaves the possibility for the development of more effective and safer compounds to use for the treatment of NSCLC.

Distinguished professor Dr. Wiley Youngs' research group works with imidazolium salts as a means to better treat NSCLC. By varying the ligand additions to the various positions of the imidazole ring, the Youngs lab has developed numerous anti-cancer compounds, of which methyl naphthalene on the nitrogen atoms of the imidazole give the best activity. While these compounds exhibit antiproliferative activity, success has been limited due to poor solubility.[3] Synthesized imidazole compounds thus far must first be dissolved into either dimethyl sulfoxide (DMSO) or cyclodextrin before testing.

Echinomycin and ethidium bromide are known compounds with planar aromatic structure that allow for DNA intercalation. Echinomycin specifically exhibits double intercalation behavior.[4] This double intercalation is of interest for the bis-imidazolium salt compounds as a possible mechanism of drug targeting. Recently, the Youngs lab has taken interest in synthesizing a bis-cation system, which consists of two napthalenes, two imidazole salts, and a linking chain between them that varies in length from 1-12 carbons. This bis-cation system produces more polar compounds, increasing the chances of aqueous solubility.

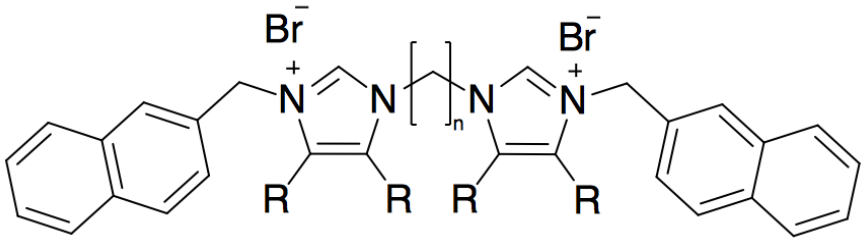

Figure 1. Displays the general structure of the bis-cation system, where n is the varying number of carbon atoms connecting the two chains and the R groups in the 4, 5 positions of the imidazole salts are defined as hydrogen.

Because the bis-cations have shown increased polarity and solubility, the purpose of this project is to test the potential intercalation of the system by means of viscosity and fluorescent intercalator displacement (FID) to determine any intercalation with DNA. Analyzing viscosity, it is hoped that these compounds increase the viscosity of the DNA, meaning the backbone has been slightly unwound to ease the strain caused by the intercalating compound.[5] FID experiments using ethidium bromide will aid in demonstrating the possible intercalation properties of the bis-cations. It is hoped that a decrease in fluorescence will be seen, implying the displacement of the ethidium bromide by the compound.[6] If this analysis proves useful in better understanding the mechanism of the bis-cation compounds, new compounds can be synthesized specific to the binding mechanism.

Methods

The bis-cation system was tested for intercalating behavior by both viscosity and fluorescent intercalator displacement (FID). The system contained linker compounds ranging from 1-12 carbons in length. For time convenience, 4 of the 12 compounds were chosen for experimentation: methyl, propyl, pentyl, and heptyl. All compounds were first

solubilized in DMSO (10%) and diluted to 1mL with Invitrogen™ Ultra Pure Distilled Water (DNAse, RNAse free).

Invitrogen™ calf thymus DNA 10 mg/mL; Thermo Fisher Scientific stock solutions were separately prepared for each testing method. For a concentration between 115 μM and 135 μM for FID, 30 mL of Tris/NaCl (5mM/50mM) buffer and 110 μL of calf thymus DNA were added to a falcon tube and vortexed. For a concentration between 225 μM and 250 μM for viscosity, 70mL of Tris/NaCl (5mM/50mM) buffer and 560 μL of calf thymus DNA were added to a falcon tube and vortexed. An Agilent Varian Cary® 100 Bio UV-Visible Spectrophotometer was used to determine the concentrations of the prepared DNA solutions. The instrument was warmed up for 20-30 minutes. The software was opened and the instrument was zeroed. Tris/NaCl (5mM/50mM) buffer was added to the blank cuvette and placed in the back of instrument as the reference. DNA solution was added to the sample cuvette and placed in the front of the instrument. After absorbance values were collected between 200 nm and 800 nm, x and y peaks were labeled on the graph and the DNA absorbance value was recorded at 258 nm. The Beer-Lambert equation was utilized to calculate the concentration of the DNA solution using the recorded absorbance and extinction coefficient (6600 M^{-1} cm^{-1}).[7]

Fluorescent intercalator displacement was conducted based on the procedure previously described by Boger et al. (2001). A HORIBA Jobin Yvon Fluoromax-4 Spectrofluorometer was used to conduct FID testing. The Fluoromax-4 was warmed up for at least 30 minutes. With no cuvette in the instrument, a lamp excitation spectrum was collected to determine the initial intensity. Nanopure water (3 mL) was added to the cuvette and an emission spectrum was collected. Both lamp and water spectra were

collected using default parameters. Nanopure water was discarded and a 120 μM DNA solution (2.997 mL) and 3 μL of ethidium bromide were added to the cuvette. The excitation wavelength was set at 510 nm and the emission wavelength range was set between 465 nm and 750 nm. An initial emission spectrum was collected before any compound was titrated. Compound was then titrated in from 0-30 μL by 2 μL additions, 30-100 μL by 10 μL additions, and 100-600 μL by 100 μL additions, and all emission spectra were recorded. A pipette was used to thoroughly mix each addition and the solution was given 2 minutes between each reading to equilibrate. Intensity values were normalized against the blank to account for the lamp intensity decreasing with each use. Normalized intensities were graphed against concentration of the solution.

Viscosity testing was based on the procedure carried out by Xia-Bing Fu et al. (2014). A viscometer and thermometer were placed into a water bath. The water bath sat atop a VWR heating/stirring plate and was kept between 29°C and 30°C throughout testing. Tris/NaCl buffer (8 mL) was placed into the large bulb of the viscometer. A bulb was used to entirely coat the large and small arms of the viscometer. Timing began as the solution crossed the line above the small bulb and ended where the solution crossed the line below the small bulb. Flow times were recorded in triplicate. The buffer was discarded and a 250 μM DNA solution (8mL) was added to the viscometer and flow times were recorded in triplicate. To the DNA, 7 separate 8 μL additions of the select bis-imidazoium cation were titrated into the solution. The bulb was used to draw solution into the small arm to collect the addition, the initial flow through was not recorded, and air was bubbled through the viscometer from the small arm to ensure complete mixing before any readings of the additions were taken. All 8 μL additions flow times were

recorded in triplicate. Triplicate trials of the buffer, DNA, and DNA and additions were averaged and normalized against the buffer.

It should be noted that extreme sensitivity associated with this technique renders viscosity data highly variable and sometimes inaccurate. Multiple factors influence the flow time: recording entry and exit into the small bulb, temperature, and thorough mixing of additions into solution.

Results and Discussion

Fluorescent intercalator displacement provides insight into the affinity for DNA of the bis-cation compounds. This technique measures changes in fluorescence intensity of a solution. When ethidium bromide intercalates between DNA base pairs, fluorescence significantly increases. Utilizing this fluorescent compound, the degree to which the titrated compound displaces ethidium bromide and binds to DNA can be determined. As ethidium bromide is displaced by the compound, fluorescence should decrease.[8] FID experimentation was conducted on netropsin for means of comparison to the compounds. Netropsin is a known groove binder that has the ability to displace ethidium bromide.[8] To ensure neither the DMSO nor water resulted in a decrease in fluorescence intensity, a 10% DMSO in water solution was used as a vehicle control.

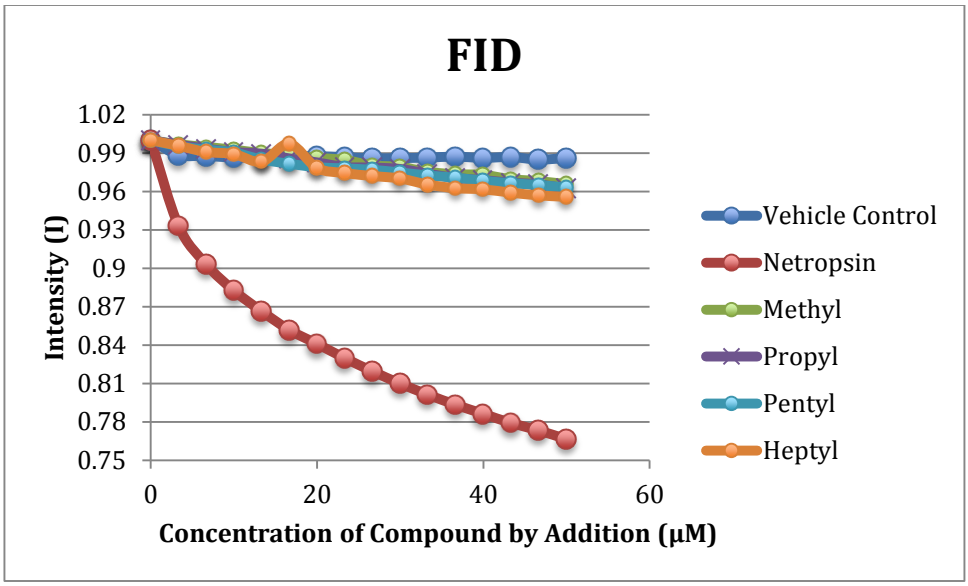

Figure 2. Figure 2 displays the graph of normalized intensities vs. the concentration of compound of the vehicle control, netropsin, and the four compounds.

All compounds show some degree of ethidium bromide displacement; however, the observed displacement is not significant as compared to the netropsin shown by the red line. No bis-cation compound comes close to rate of decreasing fluorescence intensity in comparison to netropsin. This pattern continues into the 30-100 µL and 100-600 µL additions. The orange line from the graph represents the heptyl compound and proves most effective in ethidium bromide displacement of all four compounds. Overall, the compounds did not display a stronger affinity for DNA due to their lack of displacement of the ethidium bromide.

Viscosity testing was also used to determine any intercalating behavior of compounds. DNA intercalation causes an increase in flow time. Compound intercalation causes unwinding of the DNA backbone in order to reduce strain. This base pair separation lengthens the DNA and increases viscosity.[9] Acridine orange and a 10% DMSO in water solution were used as a known comparison and vehicle control respectively.

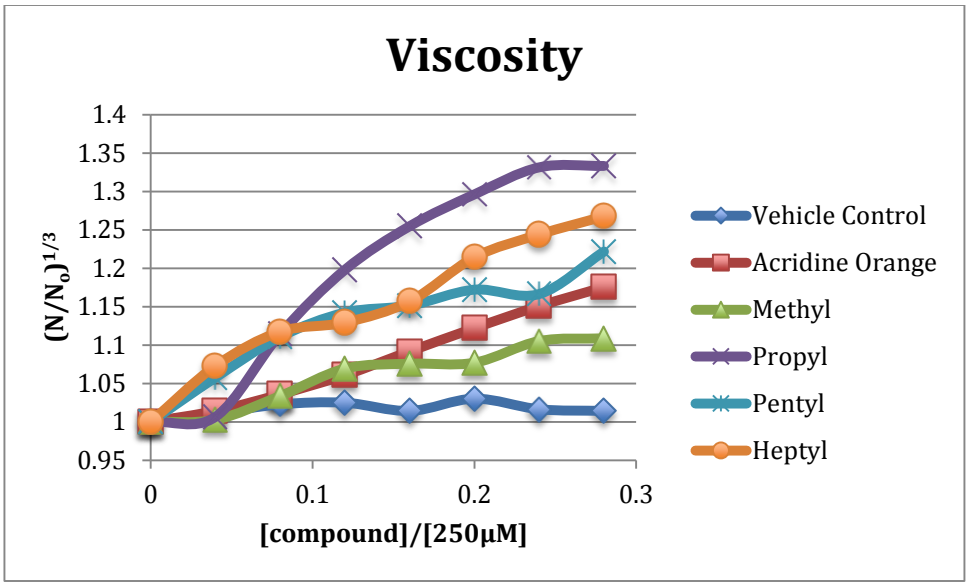

Figure 3. Figure 3 displays the graph of normalized viscosities vs. the concentration of compound by titration divided by the concentration of the DNA stock solution of the vehicle control, acridine orange, and the four compounds.

Contrary to FID, three of the four compounds displayed slower flow rates compared to the acridine orange control. These increases in viscosity suggest that the propyl, pentyl, and heptyl compounds exhibit DNA interactions to a higher degree than the control.

Interestingly, the methyl compound experienced solubility issues. Using twice the mass compared to FID, the 10% DMSO in water solution proved insufficient to completely solubilize the compound. Poor solubility of the compound may decrease its actual intercalating potential. No other compounds experienced these issues.

While FID testing concluded insignificant displacement of ethidium bromide, viscosity testing showed three of the four compounds possessing increased viscosities compared to the control. This contradicting data indicates interaction between the compound and DNA may be present, but is not strong enough to effectively displace a known intercalator such as ethidium bromide. Results from this experiment cannot

provide a concrete mechanism of DNA interaction of the compounds. Future research will likely investigate other mechanisms of action of the bis-cation species.

Conclusion

The recently synthesized bis-cation system exhibits more polar imidazolium salts, causing increased solubility. The purpose of this project was to test for possible intercalation of DNA. Experimentation was conducted by both FID and viscosity measures. The compounds displayed insignificant displacement of ethidium bromide compared to the Netropsin control. The heptyl compound exhibited the greatest intercalation of the compounds, but compared to the control its activity was negligible. Viscosity testing concluded the propyl, pentyl, and heptyl compounds showed increased viscosity times compared to the acridine orange control. Overall, the compounds exhibited some interaction with the DNA, but this interaction was not strong enough to displace a known intercalator.

Solubility remains the issue at large. The larger linker compounds exhibit increased degrees of intercalation, but their size causes solubility issues in the 10% DMSO in water solutions. A possible solution to avoid sacrificing intercalation for solubility is to test other solvents apart from DMSO and water for solubilizing compounds. Cyclodextrin may prove a viable option for compound solubility issues because of its ability to bind the compound into its lipophilic pocket.

From this experiment, it was determined that the bis-cation system causes a small degree of DNA intercalation, which proves to be too insignificant for a valid mechanism of action. In future research, it is likely the lab will test for alternative mechanisms of action rather than continuing to experiment with DNA interactions.

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Safety Appendix

Common precautions were taken the entire duration of this research. Gloves, goggles, proper shoes, working in the hoods, and an optional lab coat were used at all times during experimentation. Undergraduate students were aware of fire extinguisher and eye wash station locations.

Special attention was paid when handling and disposing of netropsin, ethidium bromide, acridine orange, DMSO, and calf thymus DNA. In this case, netropsin was mixed with ethidium bromide from FID experimentation. The combination of netropsin, ethidium bromide, and calf thymus DNA were discarded in a liquids waste container specifically for ethidium bromide titrated with imidazolium salt compounds. All tips, Kimwipes, and gloves that came into contact with ethidium bromide were disposed of in a designated solids waste container. Undergraduate students never pipetted from the ethidium bromide stock solution. From viscosity testing, acridine orange, imidazolium salt compounds, and calf thymus DNA solutions were discarded in the proper liquids waste container that did not contain ethidium bromide. Tips that came into contact with acridine orange, imidazolium salt compounds, and calf thymus DNA were discarded in a specific solids waste container.

The full properties of the imidazolium salts were unknown. Preventative measures including limited exposure, gloves, and goggles were carried out in times of working with those compounds. DMSO (10%) in water and compound solutions were discarded in a specific liquids waste container. Because it is generally regarded as safe (GRAS), cyclodextrin may prove a viable option for safely solubilizing compounds compared to DMSO.