Determining a Viable *in silico* Method For The Identification of Protein-Protein Interaction Inhibitors by Studying The Interactions of Netrin-1 DCC and UNC5

By

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A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfilment of the requirements of the degree

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Abstract

Protein-Protein interactions (PPI) are responsible for a wide variety of biological processes. The misregulation of these interactions can often times be deadly as misregulated PPI's have been known to cause cancer and other life-threatening diseases. That is why, in recent years, more time and energy has been focused on regulating PPI's. The issue faced in finding small molecule PPI inhibitors however is that PPI interfaces are very large and complex. This makes the discovery of small molecule inhibitors a challenge as the site the molecule will bind is often unknown. Currently the most common method for identifying lead compounds is high throughput screening (HTS). HTS is a costly method however and is heavily dependent on the correct selection of the chemical library. Current libraries are designed for enzyme targets and therefore may not be effective when searching for compounds that bind to the surface of a protein. The study outlined in this thesis aims at developing a more efficient and cost-effective method to test many small molecules against a PPI for their inhibitory properties. The methods used in this study employ *in silico* library screening to identify potential inhibitors. Once lead compounds are found, biological testing in the form of microscale thermophoresis and nuclear magnetic resonance spectroscopy has been completed to validate the molecular docking results. This work has identified multiple potential inhibitors, and once biotesting occurs if the hits prove valid the method outlined in this thesis will be validated.

Acknowledgements

First, I would like to thank my committee members Dr. Rebecca Davis, Dr. Jörg Stetefeld, and Dr. Francis Lin. Without your guidance, support, and lab equipment I would not have finished my thesis.

Secondly, I would like to thank Guanyu Wang for starting this project and working with me in tandem to synthesize and test our initial compounds.

Third, I would like to thank Kaitlin Isfeld, without you I would never have automated my docking and I would still be manually submitting jobs.

Finally, I would like to thank all other members of the Davis lab as well as my family and friends for the support and help through the whole graduate school process.

Dedication

This thesis is dedicated to anyone and everyone that helped me get to where I am today.

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Chapter 1: Introduction

1.1: Introduction to Protein-Protein Interactions, the Importance of Modulating Them, and the Challenges Faced

Proteins do not act as isolated species; instead they interact and communicate with other proteins through a process known as Protein-Protein Interactions (PPI's). These interactions are defined as contact between two or more proteins, i.e. electrostatic interactions, hydrophobic interactions and hydrogen bonding, that often results in a protein complex, for the purpose of serving a "function"; "functions" include but are not limited to transcription, replication and apoptosis.[1,2] This is important because these functions are integral for life when regulated correctly, however often times the "functions" are misregulated resulting in problems within the cell. Misregulated PPI's can cause a range of diseases such as cervical cancer, breast cancer, leukemia, and multiple sclerosis.[3] It is with cancer and other diseases in mind that PPI's have recently gained a lot of attention. Historically, PPI's were considered undruggable due to their complex nature and large size, however, in the early 2000's with advances in protein structure and function elucidation as well as computational analysis and new screening methods, investigating PPI's *in silico* became a viable way to make the undruggable, druggable.[4] Having PPI's as druggable targets is important because currently there is estimated to be 150,000-300,000 PPI's in the human interactome.[5] This means that there is currently an untapped market of about 300,000 targets for new drugs to help aid in the fight against cancer and other diseases.

PPI's are important to modulate, however there are currently many challenges that arise when trying to control these interactions. The biggest challenge in modulating a PPI is the size and complex nature of the protein.[6] This presents multiple issues including: 1) PPI's occur over a large surface area so small molecules cannot cover the whole interaction surface 2) Protein surfaces are generally flat, making tight binding an issue. That is why, when designing a PPI inhibitor there are many things one should consider. For example, where the inhibitor is going to bind; when dealing with PPI's, inhibitors can bind either allosterically or orthosterically.[7] Orthosteric binding occurs directly at the proteins hot spot. On the other hand, allosteric binding occurs somewhere on the proteins surface that is not the hot spot, this causes the protein to undergo a conformational change that inhibits binding.[8] This is important because while active sites are used when referring to enzymes, PPI's have a hot spot on the proteins, the hot spot is the amino acid residues that most greatly contribute to the

overall free binding energy that is taking place in a PPI. Knowing where this hot spot is and what amino acid residues are present allows for a small molecule to be designed that is the right size, shape, and has the right electronic properties in order to bind selectively and inhibit PPI's.[9]

Once the questions of how and where the inhibitor will bind are answered there are currently three strategies that can be taken to identify possible PPI inhibitors. Sheng et al.[9] identified that the first step in determining a PPI inhibitor is to determine the structure of the protein, which is commonly done using X-ray crystallography or Nuclear Magnetic Resonance (NMR) spectroscopy as seen in Figure 1.1. The next step is to determine the hot spot or other sites of interest on the protein, this can be done many ways such as NMR spectroscopy, X-ray crystallography, or alanine scanning mutagenesis. While it does not matter how one finds the druggable site of the PPI, it is important that it is found to improve the chances of correctly identifying a potential inhibitor. Once the druggable site is found there are three different strategies, as outlined in Figure 1.1, that are commonly used to find potential inhibitors. The first is using screening strategies, an example of a screening strategy is high throughput screening (HTS). HTS makes use of libraries of chemical compounds and automated equipment to quickly test the binding/inhibition of the library. The downfall to HTS is that many chemical libraries are compiled using drugs that act in conventional ways, PPI's however are very large and complex and thus fall out of the range of being a conventional drug target, meaning the small molecules in the libraries are rarely effective inhibitors. A second example of a screening strategy is virtual screening. Virtual screening uses computational methods to dock and score protein-ligand interactions, and as such can help determine if a small molecule will bind to the proteins hot spot. The second strategy discussed in Figure 1.1 is the designing strategy, an example of this is using small molecule mimics of protein secondary structure. This strategy uses small molecules that mimic α-helices, β-sheets, and β-strands to bind to the hotspot, as if they were part of the protein itself. The downfall of this method is low bioavailability and the difficulty of synthesizing the required small molecules. The final group of strategies discussed in Figure 1.1 is the synthetic strategies. Synthetic strategies involve the synthesis of diverse small molecules that can be tested against the PPI. The two biggest issues with this strategy are time and money. It takes a lot of time to synthesize the number of molecules that could be tested using a HTS method, and the money spent on both time and chemicals adds up quickly. Once a strategy has been implemented the next step is hit validation. This step involves performing bioassays such as

binding and inhibition assays to confirm the positive hits gathered at the end of screening. The last step, which is usually done by medicinal chemists, involves taking the best identified inhibitor and modifying it to see what changes can be made, if any, to make the lead molecules better inhibitors. All three of the strategies outlined to determine inhibitors show potential and have cases where they have worked. This study aims to blend these strategies into one efficient strategy.

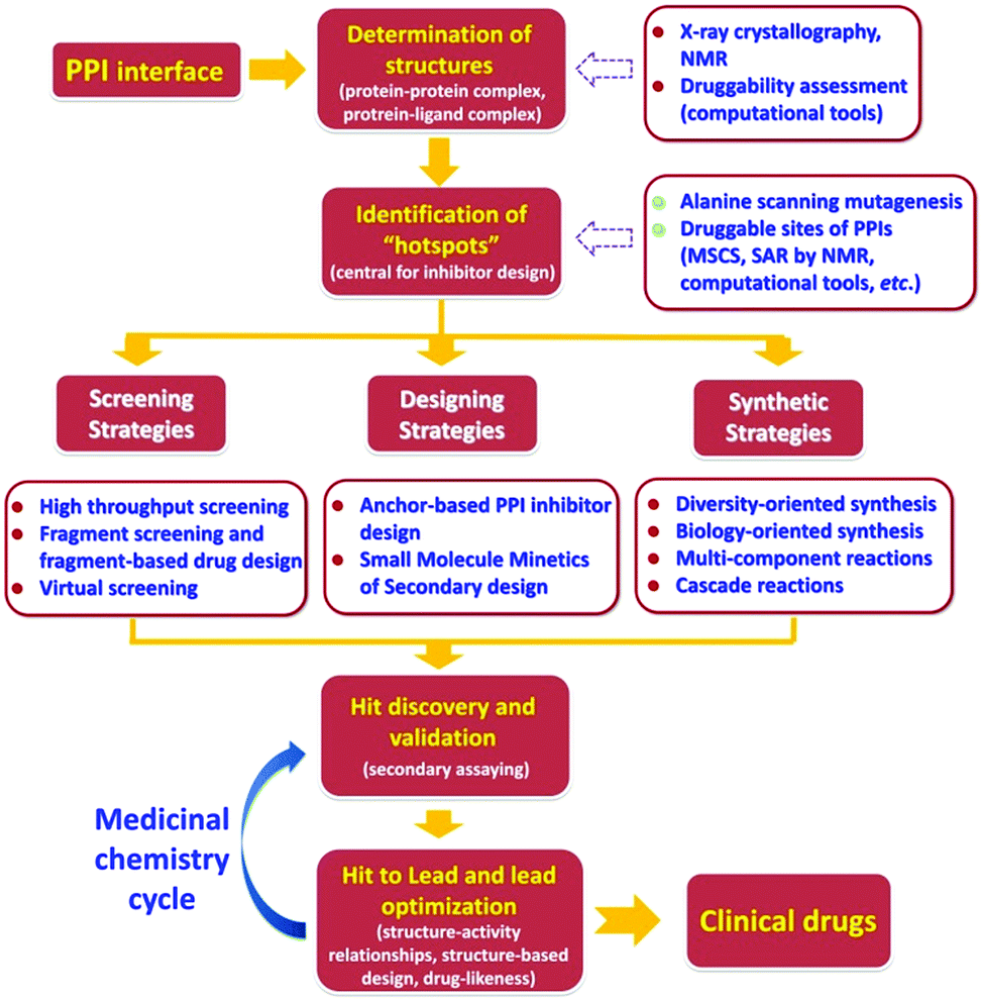

Figure 1.1. (Flow chart constructed by Sheng et. Al.[9]) Shows the steps that are taken when determining an inhibitor for PPI's.

1.2: Determination of Structures: an Introduction to Netrin-1 and its PPI's

When modulating a PPI, one must make sure there is a wealth of information available about the proteins involved as well as the PPI itself. With that in mind, my studies have focused on the PPI's of netrin-1. Netrin-1 is a secreted protein that has a variety of functions including: promoting cell adhesion, migration of leukocytes, and angiogenesis.[10] Perhaps one of its most well-known functions, however, is its role in axon guidance.[10–12]

Netrin-1 acts as a guidance cue that can attract or repel neurons. Whether netrin-1 will attract or repel a neuron depends on which receptor binds to netrin-1. When netrin-1 binds with **D**eleted in **C**olorectal **C**ancer (DCC) and neogenin it will attract the axons of nearby neurons, however, in the presence of DCC and Uncoordinated 5 (UNC5), netrin-1 will repel nearby neurons.[13,14]

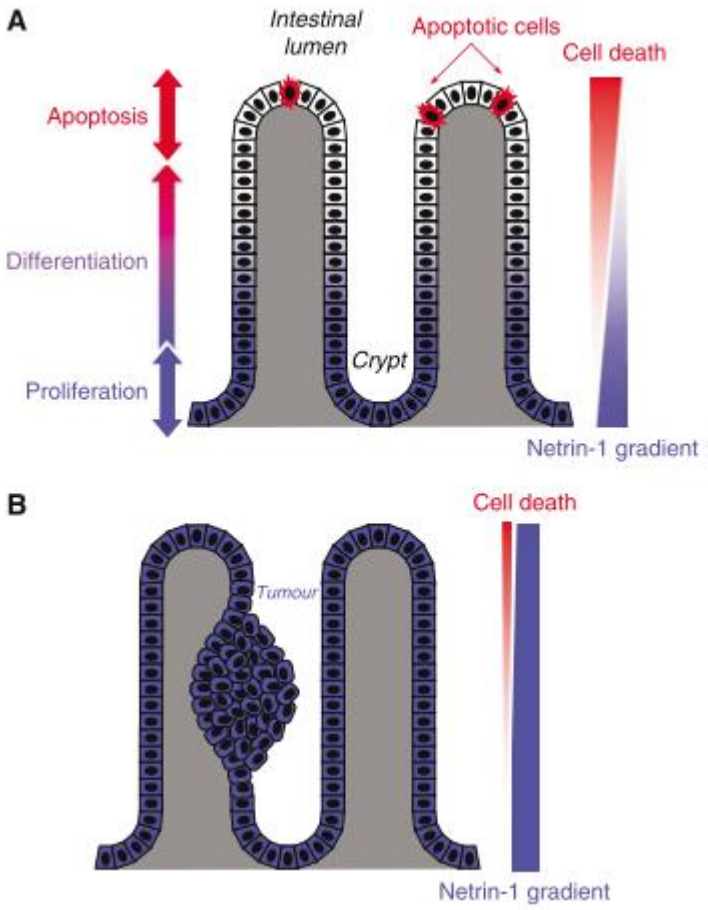

Figure 1.2. A) Shows normal intestinal villi function, whereas B) Shows intestinal villi when netrin-1 is upregulated.[15]

As seen in Figure 1.2, an excess of netrin-1 stops apoptosis and cells that express DCC in the netrin-1 rich environment will continue to grow leading to tumors. As soon as the cells leave the high concentration of netrin-1, however, they begin to die.[15] If an inhibitor were to stop the binding of netrin-1 and DCC in overexpressed regions of netrin-1 then apoptosis could occur once again. The PPI of netrin-1 and UNC5 acts in a similar manner to that of netrin-1 and DCC, whereas UNC5, in the absence of netrin-1 can lead to apoptosis[16]. So once again, if an inhibitor is introduced UNC5 and DCC will be able to lead to apoptosis and stop the progression of tumors. The ability to attract and repel axons, coupled with the

fact that misregulation of the PPI's between netrin-1, DCC, and UNC5, can lead to breast and colon cancer, to name a few, makes netrin-1 an interesting candidate to study.[13,16]

As seen in Figure 1.1, once a PPI is chosen, the structure of the protein, and complex if available, needs to be determined. For this study this was easy, as netrin-1 has been studied for many years and there are many crystal structures available. Figure 1.3 shows the crystal structure used in this study of mouse netrin with a Protein Data Bank (pdb) code of 4ove. This specific crystal structure of netrin-1 was chosen as the paper it is taken from was first to report the binding epitope for netrin-1 and UNC5.[16]

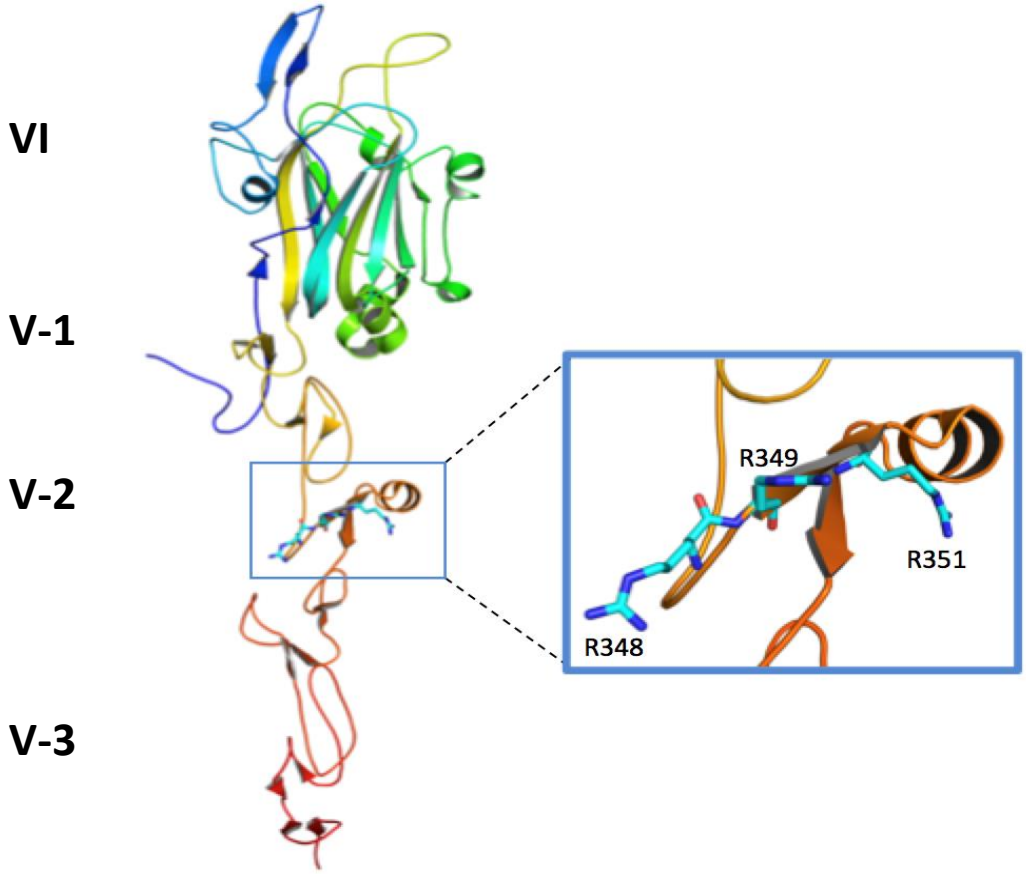

Figure 1.3. The crystal structure of netrin-1with domains VI, and V1-3 (pdb id: 4ove). Overlaid is the determined hot spot, and residues (R348, R349, R351) involved in these PPI's.[16]

One final and vital thing to note about netrin-1 is that it is a protein located in the extracellular space and has been known to bind to glycosaminoglycans (GAG's), which happen to be involved in the PPI's of netrin-1.[11] GAG's are long polysaccharides that contain repeating units of disaccharides, the GAG that netrin-1 binds to consists of repeating heparin units.[17] The importance of this will be discussed further in Chapter 3.

1.3: Introduction to Autodock4 and Genetic Algorithms

Molecular docking and structural optimization were two computational methods utilized in this study. There are many options and factors to consider when it comes to choosing molecular docking software. The first choice one must make is what type of algorithm they want their software to use. In this study a Lamarck Genetic algorithm coupled with an empirical binding free energy force field was used. This method was chosen as it has been shown to be more efficient and reliable than other genetic algorithms.[18] This is because instead of being based on Mendelian genetics it is based on the disproven Lamarckian genetics. This is best explained by Figure 1.4[18].

The right side of Figure 1.4 shows a Mendelian genetic algorithm where a parent genotype undergoes a mutation to form a child, this all occurs at the genotypic level (your data). It is then mapped to the phenotype (expression of data). The mutations that occur are small structural changes in the molecule that cannot efficiently be made during binary crossover, which in itself can most easily be described as the actual docking. The left half of Figure 1.4 shows how the Lamarck genetic algorithm works. The difference here is that the parent data is expressed first, then at a phenotypic level a local search is done. A local search takes the place of mutation and is important because it does not require local energy information and instead can look at the torsional space and search based on that. Once the first local search is complete the search process continues and step size is increased or decreased based on previous steps energy until a minimum is found.[18] Once the minimum are found, an inverse mapping function is used and the phenotype is converted back into the genotype.

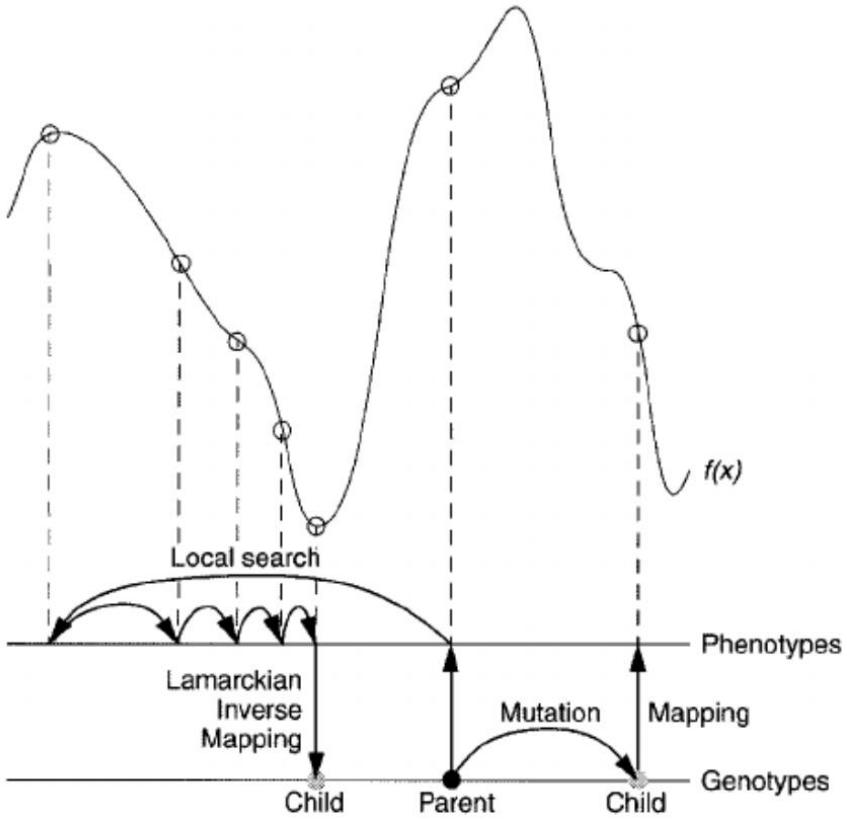

Figure 1.4. Compares a genetic algorithm (parent and right) to the Lamarck genetic algorithm (parent and left) with the fitness (f(x)) shown at the top.[18]

Molecular docking takes the structure of a protein and with a defined search space one can dock small molecules into the hot spot of said protein. The docking software will check different conformations of the molecule and then score said molecule with a binding energy. Specifically what the software does is add up the energy of all of the intermolecular interactions between the protein and the ligand, as seen in the equation $\Delta G_{binding} = \Delta G_{vdw} + \Delta G_{elec} + \Delta G_{Hbond} + \Delta G_{desolv} + \Delta G_{torsional}$[19] where $\Delta G_{binding}$ is the sum of free energy of binding, or the binding energy, the ΔG_{vdw} is the sum of free energy of the van der Waals forces, ΔG_{elec} is the sum of the free energy of all electronic interactions, the ΔG_{Hbond} is the sum of the free energies of the hydrogen bonds, ΔG_{desolv} is the sum of the free energy of desolvation, and finally $\Delta G_{torsional}$ is the sum of the free energy of the torsional strain, with all energies in kcal/mol. All molecular docking was done using Autodock4 as it is widely used and accepted, and is one of the highest cited docking software's on the market.[20,21] As described above for molecular docking, what Autodock4 does is take the uploaded protein crystal structure and has the researcher define a grid box. This grid box is generally defined as the hot spot location on the protein as this will limit the search space for the small molecules and

allow for faster calculations. After the protein is set up the user inputs a ligand and submits the docking. When docking is complete the user will get predicted binding energies, which is the sum of all intermolecular forces acting on the protein-ligand complex, as well as conformations for the way a ligand will sit in the hot spot. These two results allow one to see if the docked ligand is viable for their purposes.

1.4: Hit Validation

Once docking was completed, synthesis of the top five molecules, as outlined in Chapter 2, took place so that hit validation or running bioassays (as seen in Figure 1.1) could take place. The instrument utilized to run assays was Microscale Thermophoresis (MST), which uses temperature induced florescence changes along a titration to identify binding between two compounds.[22] The theory behind MST is based on thermophoresis which describes the force produced by a temperature gradient and how molecules respond.[22] Figure 1.5 (modified from the work of Jerabek-Willemsen et al.)[22] further describes how MST works.

Figure 1.5. shows a brief overview of how MST works. When looking at the output graph on the right side of the figure it can be observed that initially the molecules (yellow balls) are homogenously distributed in the capillary. Upon introduction of the infrared (IR) laser, a temperature gradient is established and thermophoresis takes place. The sample is irradiated with IR light for approximately 30 seconds before the laser is turned off and back diffusion of the molecules occurs. These experiments are generally completed using different concentrations of ligand molecules and based on the change in the generated graph binding can be determined.

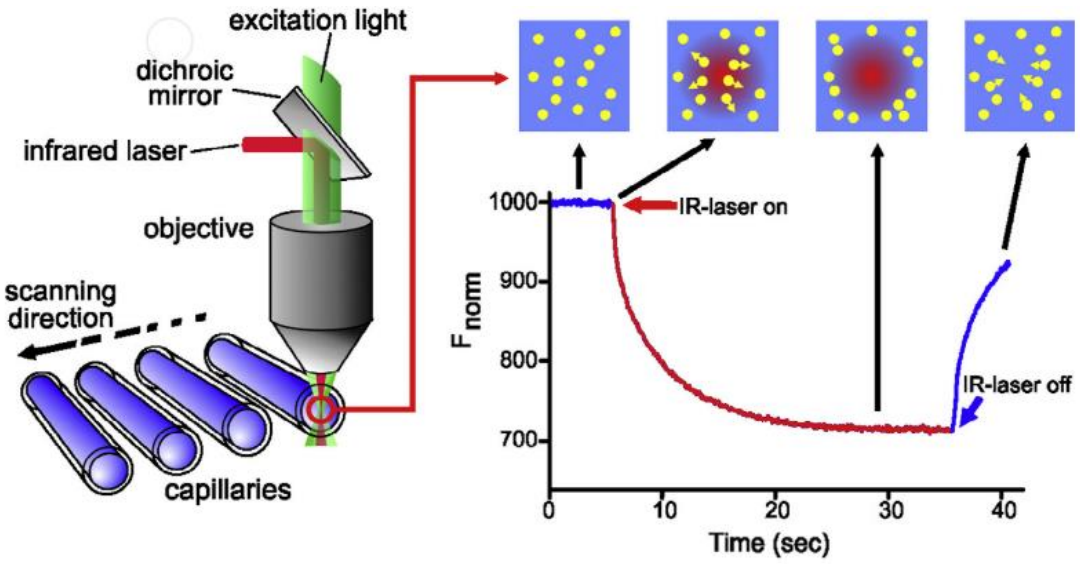

Figure 1.5. Shows how the temperature gradient is formed using IR light, and the generated graph from the systems detector.[22]

1.5: Applications in this Study

Using a combination of computational and synthetic chemistry, this study aims to develop a method of determining small molecule inhibitors of PPI's. Moreover, this study looks at regulating the PPI's of netrin-1. Regulating the PPI's of netrin-1 is important as its interactions have the potential to stop apoptosis, and subsequently start tumor growth. Therefore, this study has the potential to be used in the future when looking at therapies in colorectal cancer.

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Chapter 2: Initial Results

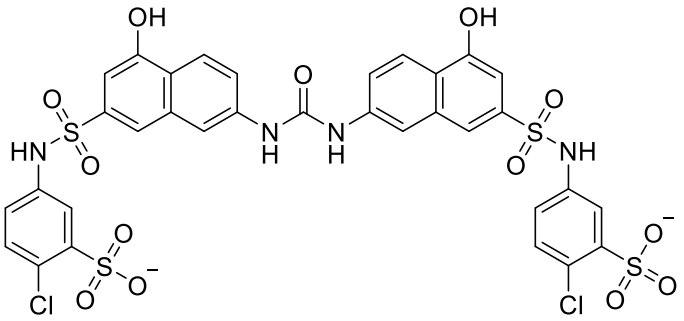
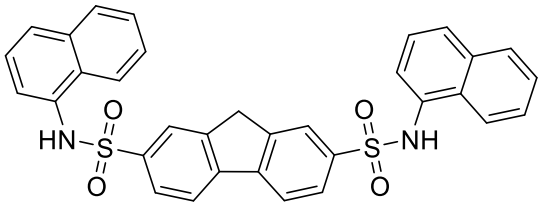
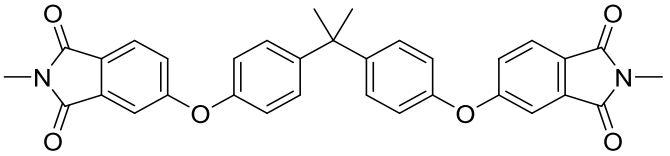
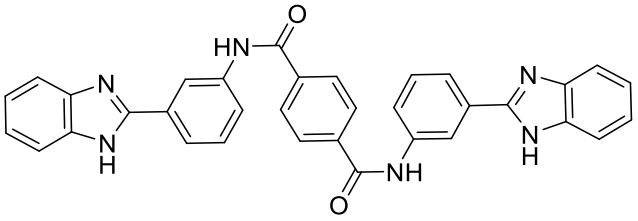
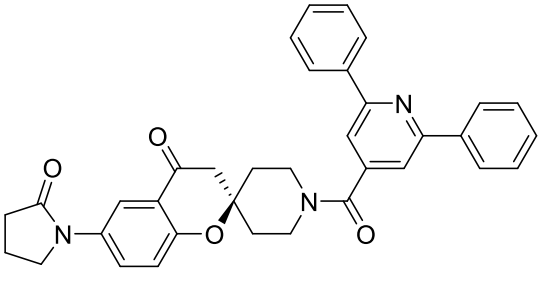
2.1: Introduction

Molecular docking has the potential to be a faster, and cheaper way for identifying PPI inhibitors when compared to HTS. This is due to the fact that you can screen significantly more molecules before moving on to biotesting. After docking one can then synthesize and test the top hits. That is what was done in the initial stages of this project, first molecular docking took place, followed by synthesis of low binding energy molecules identified from the docking. After synthesis occurred biotesting began. Using MST, which can identify fluorescence changes in molecules based on temperature change, one can tell if there is binding and can determine a dissociation constant (K_d) between a protein and a small molecule. A second method that can be used to determine binding is NMR spectroscopy, in which binding is inferred from structural changes to the protein. A combination of these techniques were utilized in this study as described further in this chapter.

2.2: Initial Library Screen

Prior to my joining the Davis lab and this project, a screening of three different compound libraries (ZINC15 in trial drug candidates, NCI diversity set II, and the Maybridge screening collection) was performed. These libraries totaling approximately 90,000 small molecules were docked into the hot spot for the netrin-1/UNC5 PPI. This docking included in the search area the three arginine residues, R348, R349, and R351, on netrin-1 as outlined in Figure 1.3. Once the PPI for this study between netrin-1 and UNC5 was chosen the next step in identifying potential inhibitors was computationally driven. The chemical libraries were docked using Autodock4, in a rigid search capacity using the Lamarckian genetic algorithm. The top 99.7%, (three standard deviations away from the mean) or 153 compounds based on binding energy were then docked using flexible residues. The search method used was the Lamarckian genetic algorithm with the scoring method discussed in Chapter 1. The residues that were made flexible were the three arginine residues outlined in Chapter 1 as being important in the netrin-1/UNC5 hot spot. The reason to do multiple rounds of docking is that rigid docking is a good starting point but to get a more accurate representation of the binding energy flexible residues are needed. The top five hits based on lowest binding energy from the second round of docking were chosen to be synthesized and then subsequent biotesting could occur. Table 2.1 shows the top five hits, their binding energies in kcal/mol, and their compound identifier.

Table 2.1. The top five docking hits that were docked into the V-2 domain of netrin-1, structures shown are assumed to be at physiological pH.

Compound identifier	Binding energy (kcal/mol)	Molecular structure
ZINC000169305875	-16.70	
BTB13627	-12.71	
BTB13885	-12.33	
NCI_61610	-12.36	
ZINC000042807218	-12.21	

One thing to note about the top four hits is that they all have some similar qualities. The top four structures all have multiple hydrogen bond donors and acceptors, they all have multiple aromatic rings, and finally they have a plane of symmetry. While slightly different,

hit number five (ZINC000042807218) also shares a couple of these similarities which could indicate that some of these properties are more important than others when it comes to docking for this PPI. Another thing that all of these structures have in common is how they sit in regard to netrin-1. Each of these five structures sit in front of the arginine residues and have interactions with all three residues as seen in Figure 2.1. The reason for these similarities could be that the three arginine residues in netrin-1 with which these compounds interact are arranged linearly so in order to bind more effectively the inhibitors must also have some linearity to them.

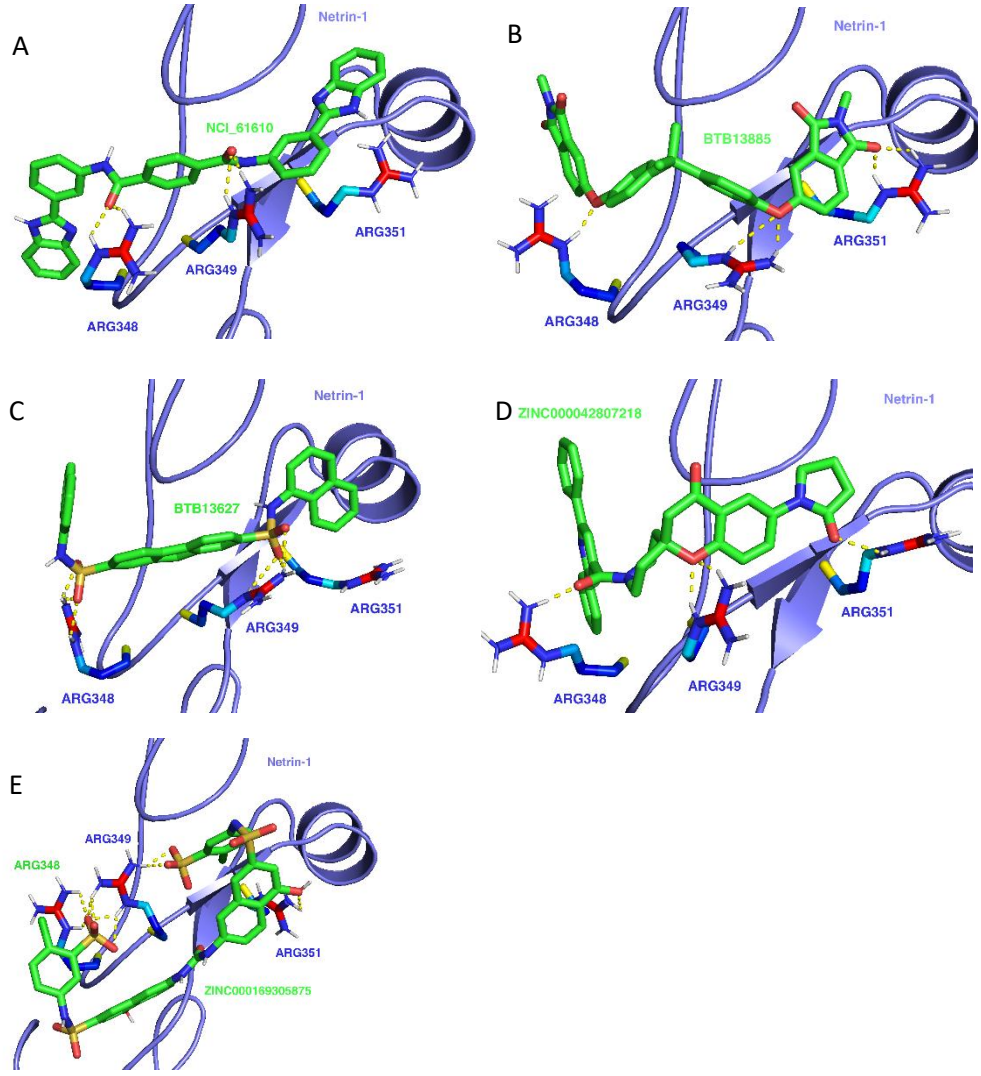


Figure 2.1 Arginine's 348, 349, and 351 (blue) interacting with the top docking hits (green) A) NCI_61610 B) BTB13885 C) BTB13627 D) ZINC000042807218 E) ZINC000169305875.[1]

2.3: Synthesis of Compounds

While five hits were pulled from the docking results, and although they all have literature methods of synthesis, only four compounds were synthesized.

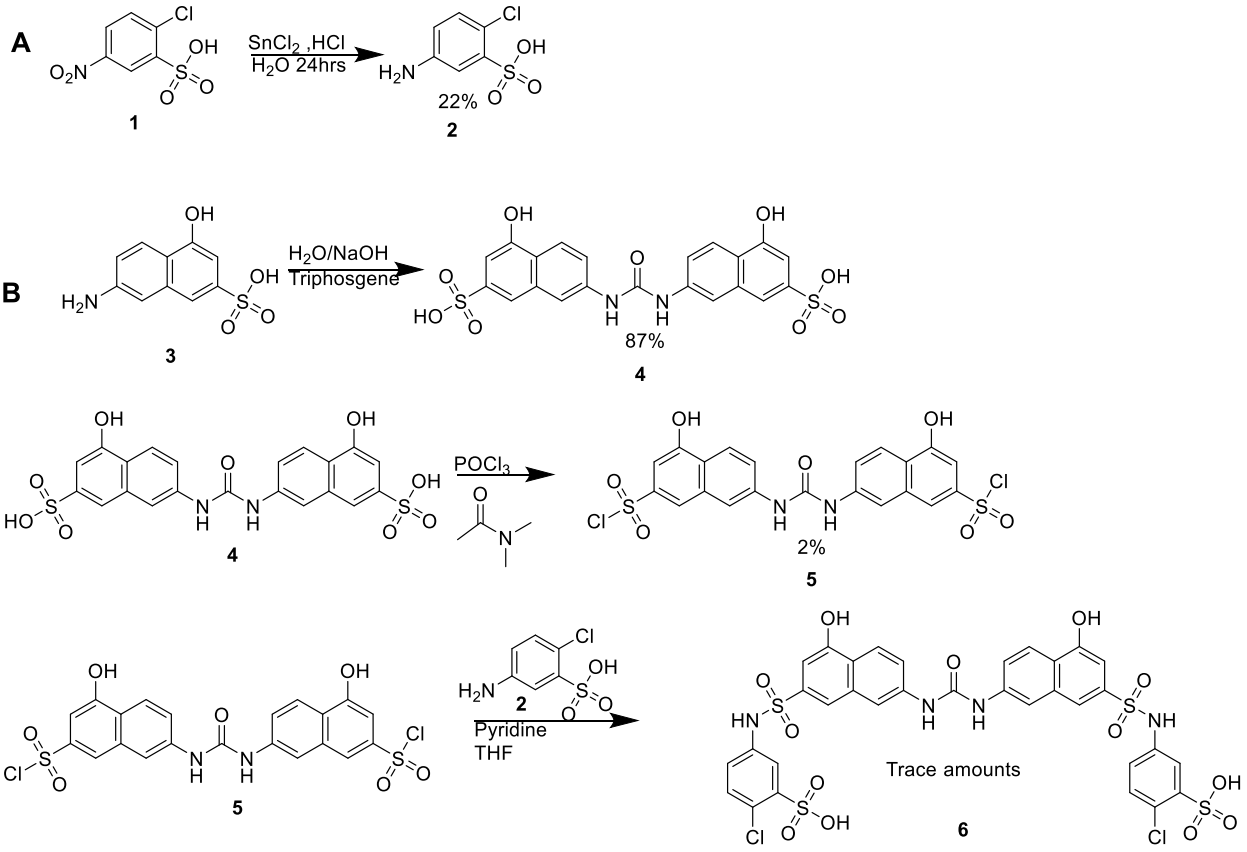


Figure 2.2. A) Synthesis of the compound needed in the final step of the overall synthesis of ZINC000169305875 B) Shows the synthesis of ZINC000169305875. Values shown are isolated yields

The compound ZINC000169305875 was synthesized using methods from multiple literature sources.[2-4] To start, ligand **2**, which is required in the final step of the synthesis as seen in Figure 2.2a was synthesized. This was a simple aromatic nitro group reduction to form an amine. Next, the urea backbone was generated using a nucleophilic substitution of 7-amino-4-hydroxy-2-naptholsulfonic acid (**3**) on to triphosgene. This substitution occurs twice before completion which is why three equivalents of **3** was used. After the substitution, the sulfonic acid was activated to a sulfonyl chloride. This was followed again by a nucleophilic substitution, this time replacing the chlorine on the sulfonyl chloride with **2**. Once again this

was allowed to occur twice before giving trace amounts of the desired product, which unfortunately was not enough for biotesting.

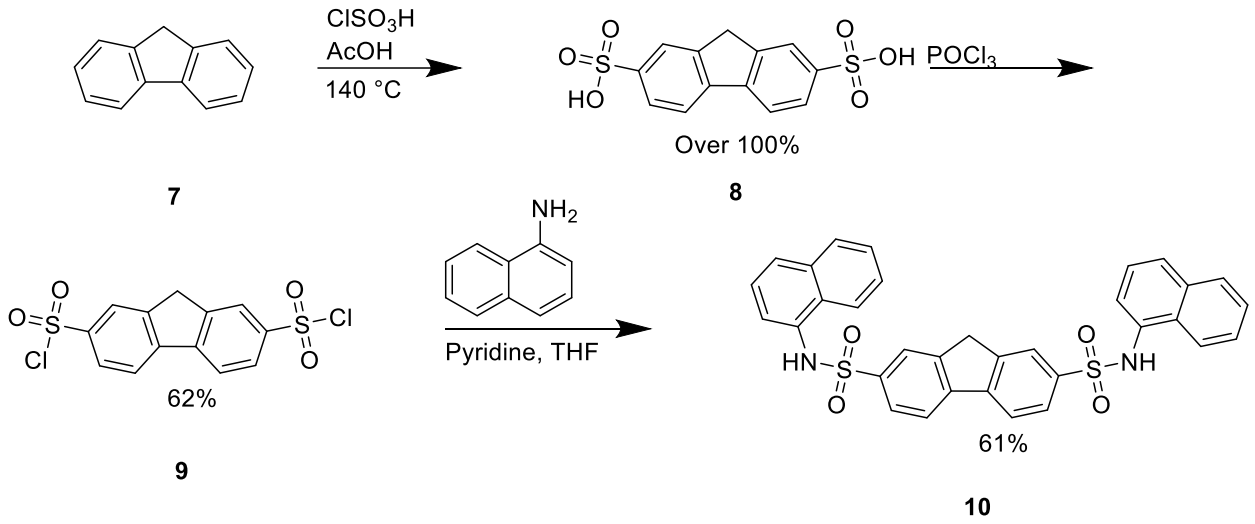


Figure 2.3. Synthesis of BTB13627. Values show isolated yields, the crude mixture of **8** was used which is why the reported yield is over 100%.

BTB13627 was successfully synthesized starting with a standard sulfonation of fluorene **7**. The product obtained from the sulfonation was impure, as seen by the yield above 100%, however, the product was then subjected to a suspension in phosphoryl chloride to successfully activate the sulfonic acid. The final product was then synthesized after the reaction of **9** with naphthylamine.

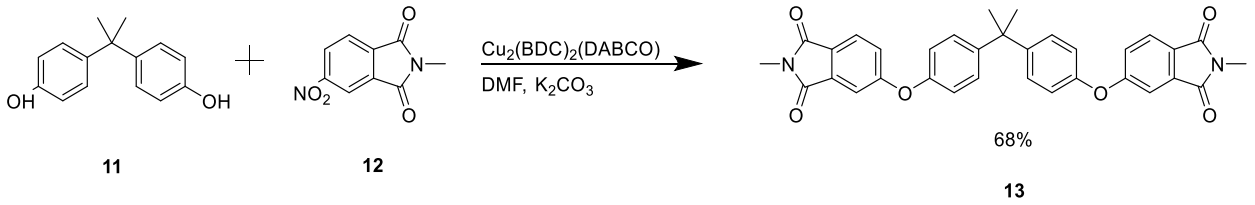


Figure 2.4. Synthesis of BTB13885. Yield shown is the isolated yield, Figure 2.5 shows the structure of the copper catalyst, $Cu_2(BDC)_2(DABCO)$.

BTB13885 was synthesized in a single step using a copper catalyzed nitroarene coupling reaction of **12** with phenol **11**. The potassium carbonate was used as a base in this reaction to deprotonate the alcohols of the phenols in **11**.

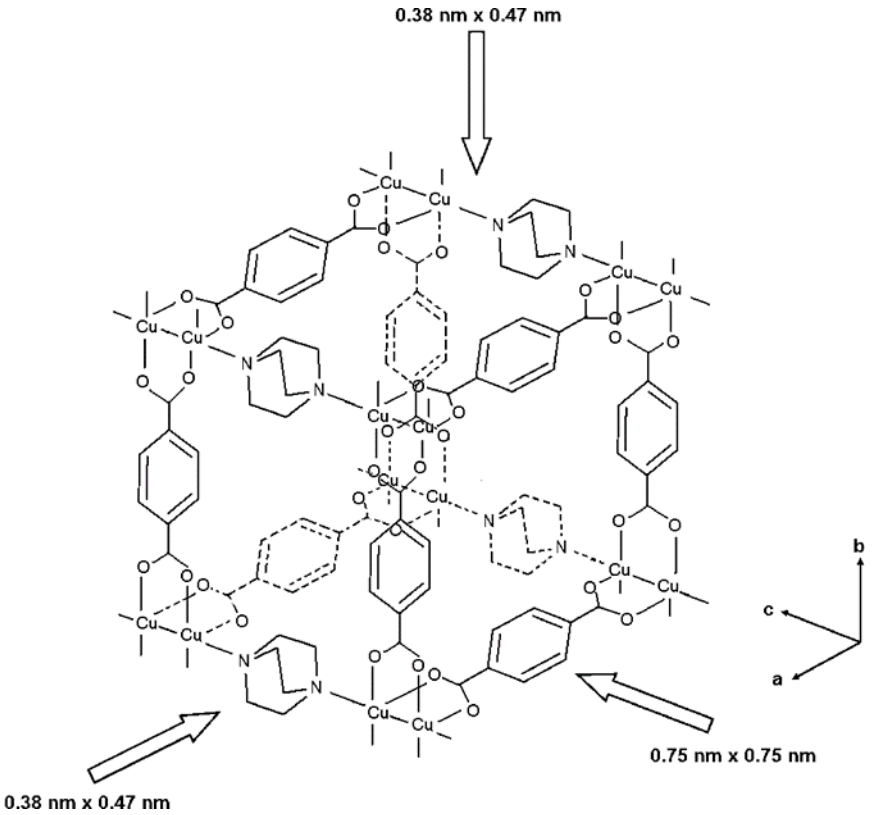


Figure 2.5. The structure of the metal organic framework of the copper catalyst used in the synthesis of BTB13885.[5]

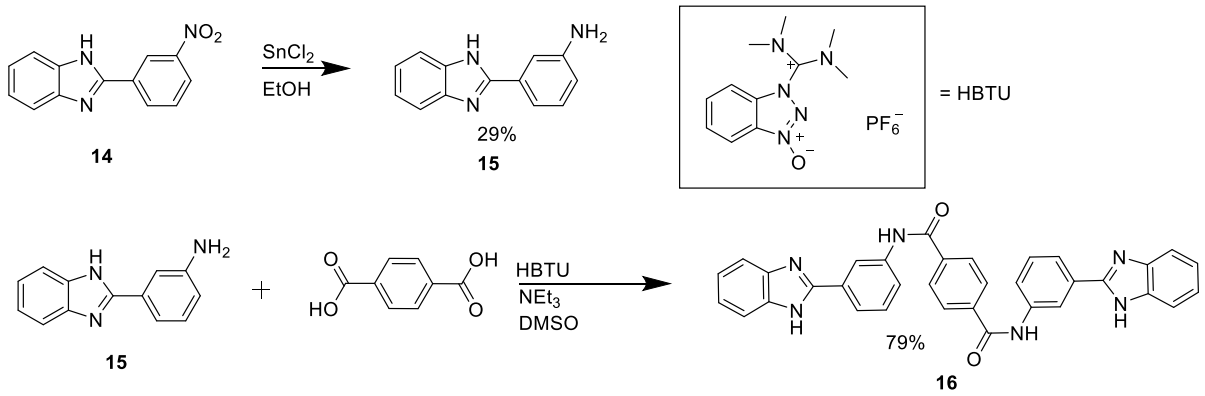


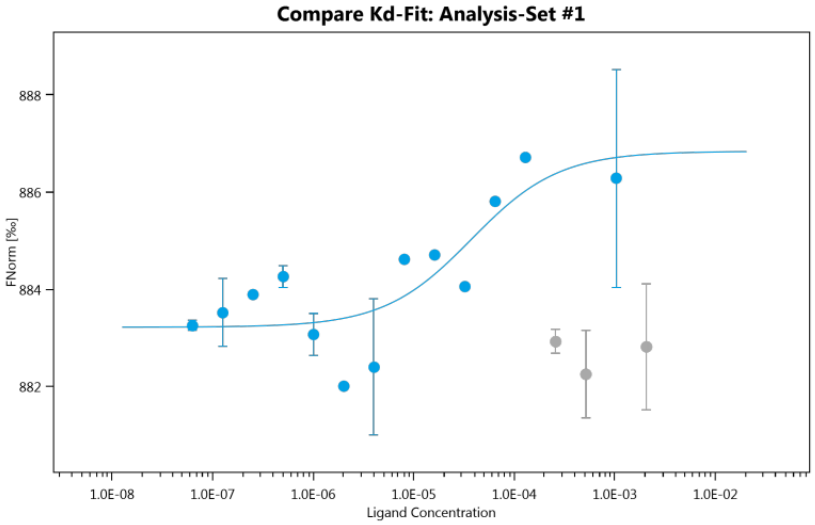
Figure 2.6 Synthesis of the amine required for the coupling reaction and the overall synthesis of NCI_61610, the values show isolated yield.

2-aminobenzimidizole (**15**) was synthesized using a simple tin chloride nitro group reduction. The compound NCI_61610 was synthesized using the standard amine to amide HBTU coupling reaction. HBTU or **H**exaflourophosphate**B**enzotriazole**T**etramethyl**U**roniom is a mild activator and works great for forming hydroxybenzotriazole leaving groups. The desired product was isolated in 79% yield.

2.4: Biological Testing and Results

MST and NMR spectroscopy were used to assess the binding of the synthesized small molecules. First, MST was run, due to the fact that MST will not only identify if there is binding present, but also can determine the K_d. This is important because from the K_d the affinity of the protein and small molecule can be determined. Using chicken netrin-1 (chNet1 ΔC) with a fluorescent dye (Alexa Fluor 647), and one of the synthesized compounds in a standard MST buffer (50 mM trisaminomethane (Tris) pH=7.4, 150 mM NaCl,10 mM $MgCl_2$, 0.05% Tween-20), MST was run on two of the synthesized compounds. Compound ZINC000169305875 could not be run since only trace amounts were recovered from the reaction and compound BTB13627 was not run as it was not soluble in the standard buffer we were using. To run the samples, a NanoTemper Monolith NT.115 and Monolith NT.115 capillaries from NanoTemper Technologies was utilized. Using MO.Control and MO.Affinity Analysis software on the MST in the Stetefeld Laboratory, a couple different assays were run. The first MST study completed used a 0.0414 M stock solution of NCI_61610 that was then titrated into a solution of netrin-1 bound to UNC5. The next assay run was a solution of netrin-1 and NCI_61610 with varying concentrations of UNC5 titrated in. Figure 2.6 shows the results and K_d for both compounds. Finally, the assays were run in triplicate to minimize error in sample preparation and give a more accurate overall picture.

A



B

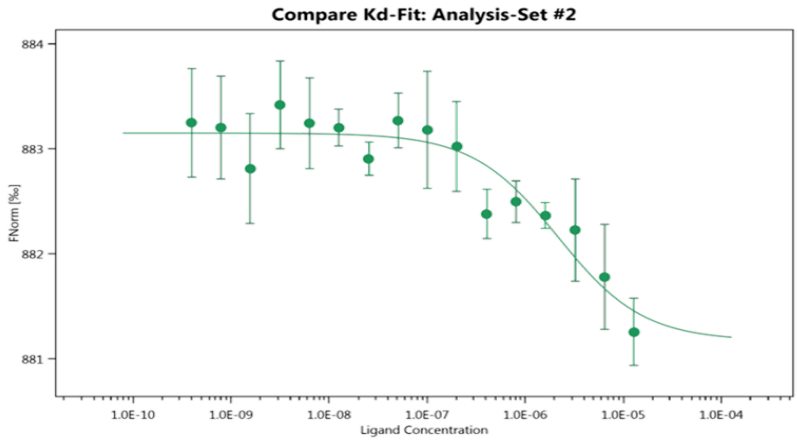
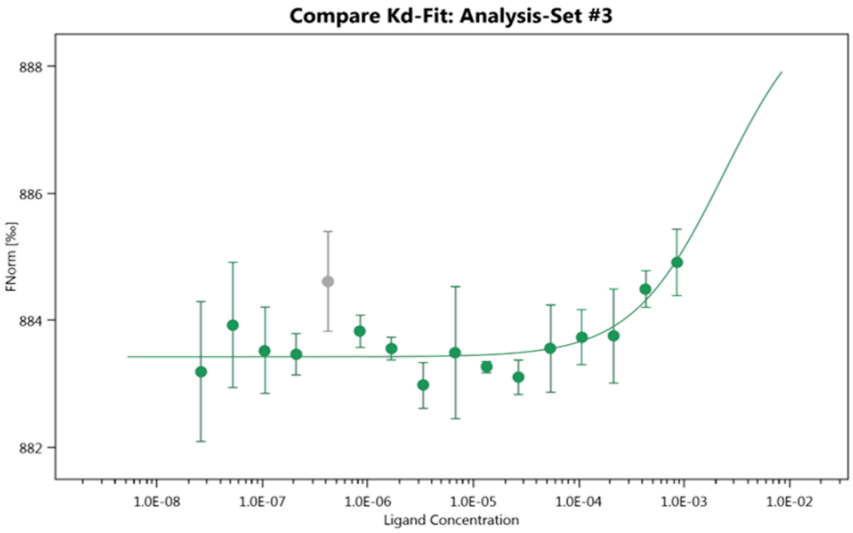


Figure 2.7. A) Shows the result of titrating NCI_61610 into a solution of netrin-1 and UNC5, the K_d for this test was determined to be 3.7485E-05 M B) Shows the result of titrating UNC5 into a solution of netrin-1 and NCI_61610, the K_d for this test was determined to be 2.1556E-06 M.

As seen in Figure 2.6, the dissosciation constant for the second assay (titrating in UNC 5) has a lower value, meaning that it is a better binder. However, the dose response curve is negative leading to the conclusion that binding of NCI_61610 was occuring. The same tests were then completed on a 0.0172 M stock solution of BTB13885. The results for these test are shown in Figure 2.7.

A



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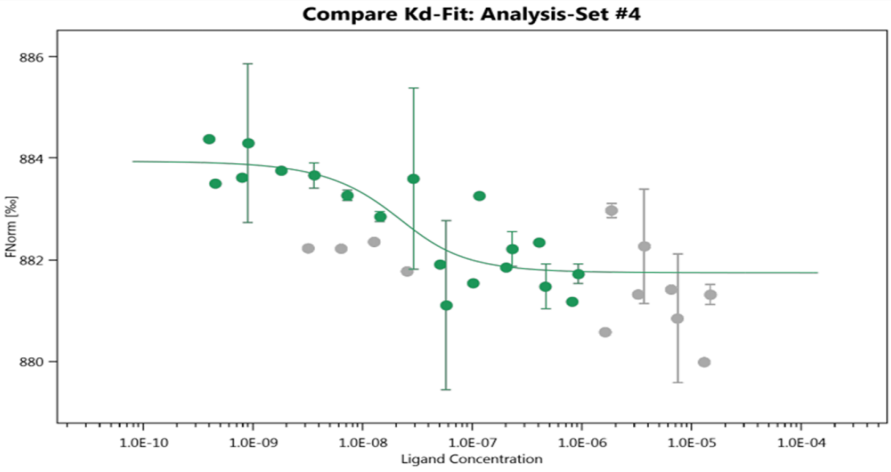

Figure 2.8. A) Shows the results of titrating BTB13885 into a solution of netrin-1 and UNC5 the K_d was determined to be 0.0023574 M. B) Shows the results of titrating UNC5 into a solution of netrin-1 and BTB13885, here the K_d was determined to be 1.0456E-08 M.

These results, illustrated in Figure 2.8, were similar to NCI_61610 in that when titrating in UNC5 the dose response was negative. One thing to note in these results however is the light gray points on the graph. These are data points that were left out of the calculation of the K_d value. In MST it is standard practice that points can be omitted. The reason for omitting points here was a combination of too low of a signal to noise ratio, capillaries that were potentially contaminated so aggregates of the protein occurred in the test, or the dose response of the capillary being too small. At first these results were thought to be promising as when adding the compounds to a solution of netrin-1 and UNC5 there was binding and that when the compounds bind to netrin-1 it interrupted the netrin-1/UNC5 PPI. Finally, as a control an assay was run of only the synthesized compounds and UNC5. This assay showed

no binding which confirmed in the other results that the compound was in fact binding to netrin-1.

It was suggested that due to the number of points which had to be omitted, that the MST results were open to some interpretation and that while there could be binding, it was not necessarily inhibiting the PPI, and the binding that was shown had the potential to be anywhere along the protein. So, to get a better picture of the binding some NMR spectroscopy studies were completed. The samples were given to our collaborator Dr. Vu To in the Stetefeld lab who found that based on the NMR spectroscopy experiment performed no significant binding was found to be occurring in the hot spot. This was determined by tagging the hot spot and looking for changes in the NMR spectroscopy once the compounds were added.

2.5: Conclusions

After synthesizing four compounds and testing two using both MST and NMR spectroscopy it was found that no significant binding that was occurring between the small molecules and the netrin-1 hot spot. This means that while there may have been some slight binding of our compounds to netrin-1 again this was open to interpretation, and if there was binding it definitely was not happening at the site we were interested in. This caused a decision to be made that more docking should be completed in order to determine new compounds that would inhibit the PPI. Chapter Three outlines how the next docking completed differed from the first set of docking. Chapter Three will also outline more about netrin-1 and how the structure as well as the environment netrin-1 resides in played into determining new structures to dock.

2.6: References

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Chapter 3: Molecular Docking

3.1: Introduction

After receiving the NMR spectroscopy results indicating no binding of the synthesized compounds to netrin-1, more research into the binding environment of netrin-1 took place. In the body netrin-1 is a secreted protein that is found in the extracellular matrix, and it binds to GAG's that are comprised of sulfated heparin. The binding of heparin to netrin-1 takes place in the V-2 domain of netrin-1 and it does not have any interactions with three arginine residues discussed in Chapter 1. Therefore, the native heparin ligand does not interact with the arginine residues that are determined to be important in the binding of UNC5 to netrin-1.[1] However, it does sit in the same V-2 domain of netrin-1, sitting just below the pocket where the three arginine residues reside, this was important because now instead of trying to block the pocket of arginine residues, a location for possible competitive inhibition was identified. This is when it was decided to dock mimics of the native ligand in the allosteric site to predict if that could inhibit the PPI.

3.2: Docking Heparin Mimics

After getting the negative results from the MST and NMR spectroscopy studies for the initial batch of compounds, the plan had to be reworked. It was here that it was brought to light that the hot spot where the docking was taking place had a native heparin dimer sitting in or next to it, which has been theorized to help binding of specific receptors, in this case DCC.[2] With all of the above in mind, more molecular docking took place. The first round of docking that took place after this realization was done on previously docked compounds that were then run through a similarity search and had bioisosteres generated of them. What a similarity search does is take a lead molecule, in this case heparin, and compares the size, shape, and electronic properties to query molecules. The search software then generates an output file that ranks the query molecules in order of similarity to the lead molecule. This output was then taken and bioisosteres were generated for some of the top hits. The generation of bioisosteres took place using a software that takes a lead molecule and systematically changes predefined segments of that molecule to ones with similar size and/or electronic properties. This next round of docking took place using the same computational docking methods, i.e. Autodock4, but with the knowledge that netrin-1 binds to long chains of sugars in the body, the approach of how small molecule libraries where chosen was changed. This time instead of docking libraries of random compounds, libraries of carbohydrates were docked, the results of which are discussed below.

Our collaborators in the Stetefeld lab provided us with an unpublished crystal structure of netrin-1 that contained a heparin sulfate hexamer as seen in Figure 3.1a. With the provided crystal structure, it was decided that instead of a heparin hexamer the heparin dimer as seen in Figure 3.1b should be docked near the three arginine residues to get a baseline for the binding energy and the docked ligand conformation compared to in the crystal structure as seen in Figure 3.2.

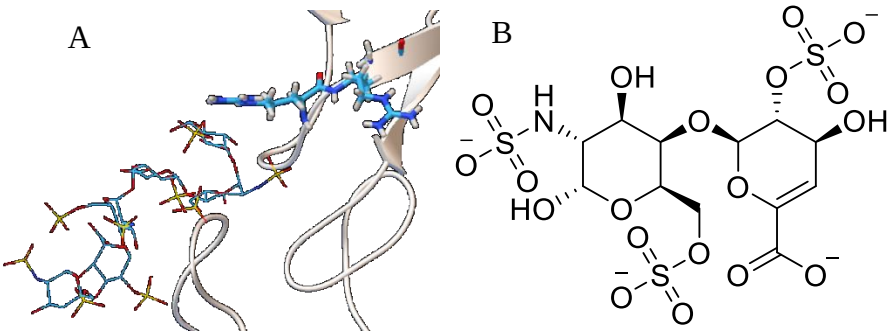

Figure 3.1. A) The unpublished crystal structure of netrin 1 with the heparin hexamer (blue) and the three arginine residues (bold blue). B) The 2D representation of the heparin dimer extracted from an unpublished crystal structure.

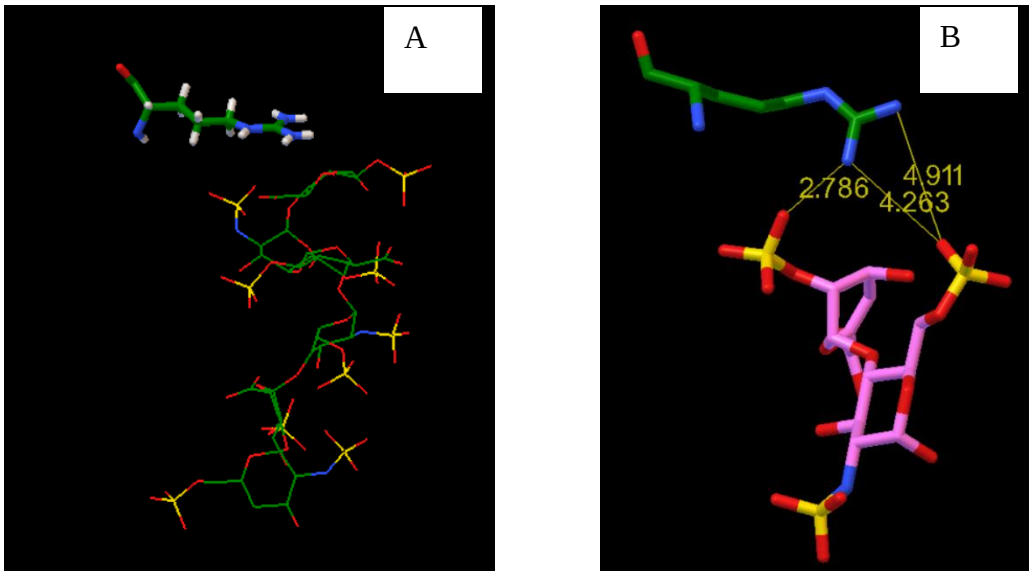

Figure 3.2. A) The provided netrin-1 crystal structure heparin hexamer with respect to arginine 348 (bold). B) The docked heparin dimer with respect to arginine 348, all distances are in angstroms.

Figure 3.2 shows that both the heparin dimer and the heparin hexamer sit in relatively the same position. The binding energy is important as the heparin is the native ligand in the PPI, so in order to pick a better inhibitor candidate a lower binding energy is needed. The

binding energy of the heparin dimer was -6.87 kcal/mol. This means that any structure that had a lower binding energy is potentially a good candidate as an inhibitor and anything with a higher binding energy is likely a poor inhibitor. The reason that a lower binding energy is important is because the small molecule inhibitor could potentially displace the heparin causing inhibition. That being said a second important criteria for good inhibitors are how the small molecule sits in the site of interest. The heparin dimer that was docked in this study also sits where the heparin dimer is in the crystal structure given to us by our collaborator. With this in mind it was decided to dock mimics, or synthetic compounds that mimic the characteristics of heparin, as well as other hexoses to see what binding energies and conformations could be found.

3.3: Docking Carbohydrate Library

With the information that netrin-1 binds to heparin in the V-2 subdomain docking commenced on a library of 470 hexose dimers that were taken from the Carbohydrate Structure Database.[3] The library was analyzed using rigid docking and the same Autodock4 methods as used in Chapter 2. These compounds all had higher binding energies by between 0.64-6.12 kcal/mol then the native heparin ligand in the protein hot spot. These structures also tended to move around the protein more whereas the heparin dimer would sit in one place when the conformation changed, meaning they were poor inhibitor candidates as they would not provide high site selectivity when binding to our protein.

With the poor binding results, it was back to square one. The native heparin ligand was once again used to see if it could achieve better docking results. As discussed below the native ligand was used in making a library of bioisosteres. Using the ROCS and EON software from Open Eye Scientific on previously docked molecules (ZINC15 in trial drug candidates). The hope here was that a molecule which was previously docked would have a binding energy less than the native ligand but higher than the compounds identified in the top 0.3% of the initial docking. What ROCS does is score a library of compounds by 3D shape similarity to a pre-stated compound, in this case the native ligand, using a Tanimoto score.[4] These results are then taken, and EON is run, which looks at the same molecules and scores them based on the similarity of their electrostatic properties to the lead molecule.[5] Both software's reordered the input of the ZINC15 in trial drug candidates and interestingly the top nine hits from both of these software's were the same nine molecules in both cases. These top nine compounds, pictured in Figure 3.3, were then taken for further docking, this time

using 100 conformations instead of the ten which had been previously used. In Figure 3.3 the compounds have (C#) associated with them. The C stands compound, and the number indicates the order in which the ROCS/EON software's raked the compounds in the library with respect to the heparin pictured in Figure 3.1b. This means that C1 was determined to be most similar to the heparin dimer with respect to shape and electronic properties.

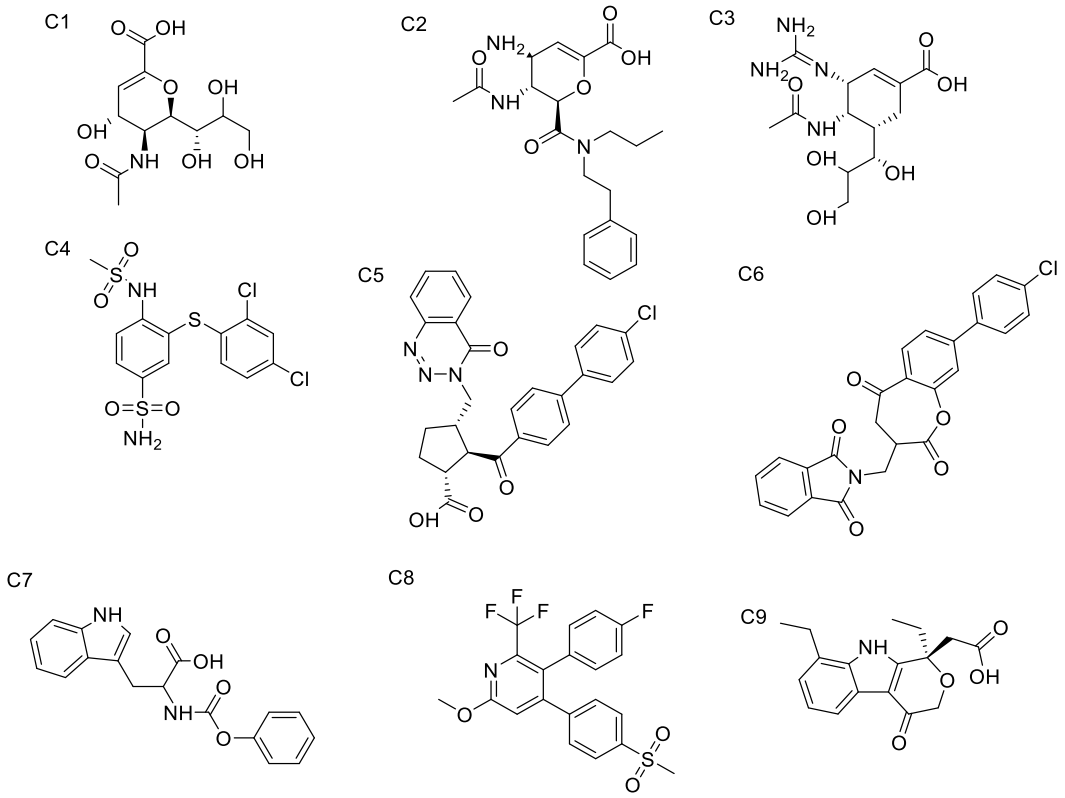

Figure 3.3. The top nine compounds identified by the ROCS and EON software's.

Table 3.1. The binding energies of the top nine hits identified from the ROCS/EON software.

	Binding Energy (kcal/mol)
C5	-7.85
C6	-7.10
Native Ligand	-6.78
C7	-5.62
C8	-5.20
C9	-5.13
C4	-4.87
C2	-4.00
C1	-3.28
C3	-2.45

Table 3.1 shows the binding energies of the top nine hits. One thing to note about these structures is that they also interacted with the same residues as the native ligand. This docking resulted in two compounds that had lower binding energies than the native ligand as seen in Table 3.1 and Figure 3.4.

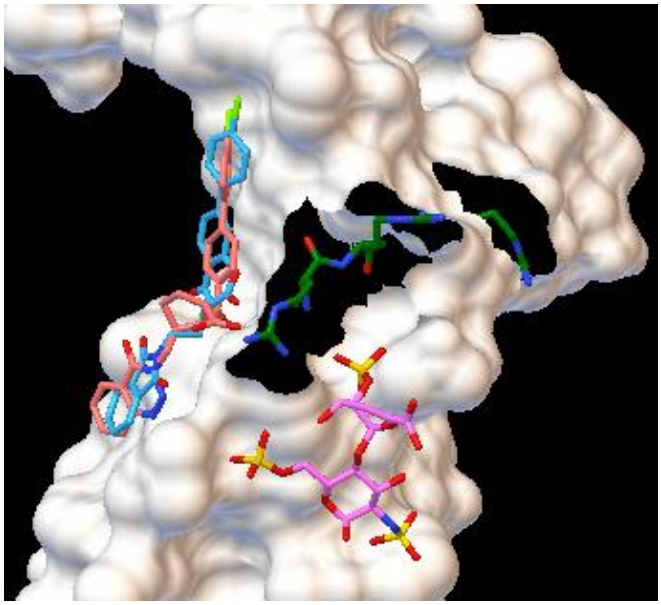

Figure 3.4. The lowest energy binding position of Hit 5 (red) and Hit 6 (blue) with respect to the native ligand (pink) and the three arginine residues in the hotspot (green).

These compounds did not sit near the native heparin ligand as seen in Figure 3.4, so they were then taken and run through the BROOD software from Open Eye Scientific. This was done to generate more potential inhibitor candidates, because what BROOD does is using a provided library of fragments it generates bioisosteres of the specified molecule that have similar electrostatic properties.[6] There are two different types of bioisosteres, there are classical bioisosteres which contain simpler structural features, and similar steric and electronic properties to the replaced portion of molecule; there is also neoclassical bioisosteres which replace a portion of a molecule with something that is a different size, shape, and has different electronic properties.[7] Once the BROOD software had finished running docking commenced again as the results provided 454 new inhibitor candidates. As before this resulted in two compounds that had lower binding energies then the previous compounds.

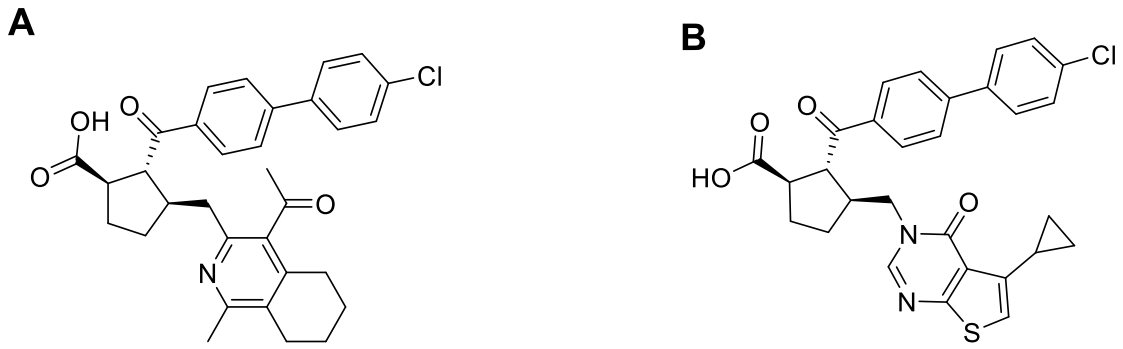

Figure 3.5. A) Shows compound 1 which has a binding energy of -9.30 kcal/mol. B) Shows compound 2 which has a binding energy of -8.76 kcal/mol.

Figure 3.5 shows the two structures with the lowest binding energy that were identified by the docking of the BROOD generated bioisosteres. Both of these compounds are lower in energy then the native ligand which has a binding energy of -6.78 kcal/mol. The compounds in Figure 3.5 are great candidates based on binding energy alone, however as seen in Figure 3.6 these structures do not sit in the same place as the native ligand, or even where the structures from which these bioisosteres were generated sit, meaning they may not displace the native ligand from the binding pocket, however that is not necessarily required to prevent the PPI's of netrin-1. Also, there is currently not a synthetic route available to make them and having three stereocenters makes designing a synthetic route very difficult. For these reasons even more docking commenced.

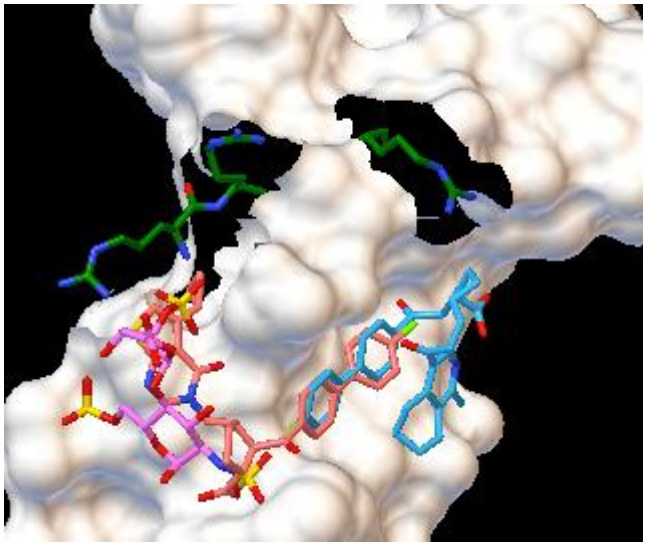

Figure 3.6. Representation of where both compounds 1 (blue) and 2 (red) from Figure 3.5 sit compared to the heparin dimer (pink) and the three arginine residues (green).

Due to limited access to the lab, the synthesis of these compounds was postponed, and further docking took place. The most recent library to be docked was structures of commercially available drugs, the thought here was that drug repurposing has worked in the past to identify new ways of treating medical issues, so maybe it can work in the identification of small molecule inhibitors. These structures were once again taken from the ZINC site. Table 3.2 shows the top 8 lowest binding energies from this round of docking, and Figure 3.7 shows that these structures sit in the same pocket as the native ligand.

Table 3.2. The common names and binding energies of the top 8 hits after the latest round of docking

Common name	**Binding energy (kcal/mol)**
Tobramycin	-18.01
Neomycin	-17.40
Paromomycin	-15.04
Arbekacin	-13.56
Gentamicin	-12.39
Hyaluronic Acid	-12.19
Plazomicin	-12.12

One important thing to note is that of the 8 lowest binding energies, five of the compounds are aminoglycoside antibiotics. This will be touched on more in Chapter 4.

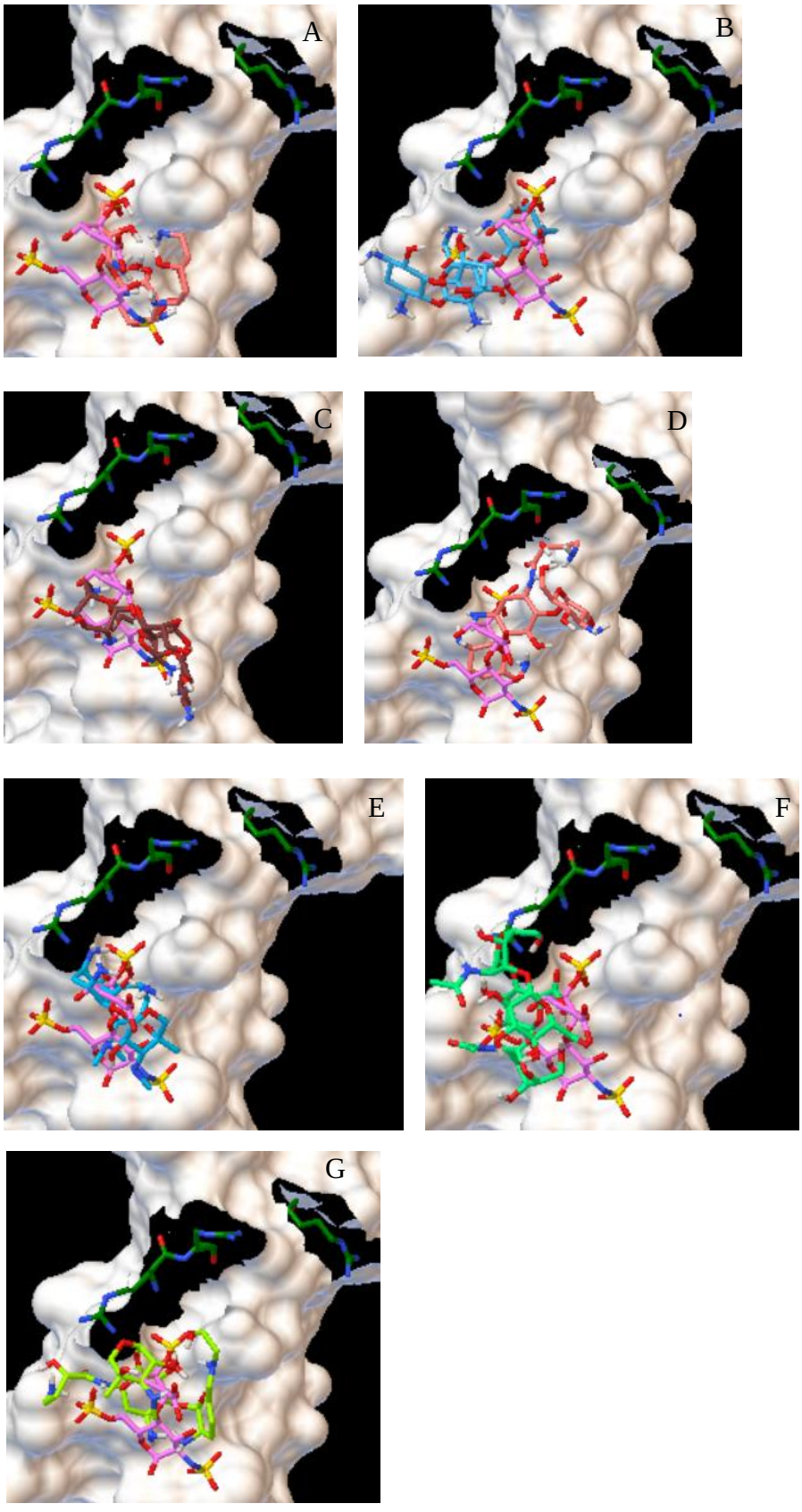

Figure 3.7. Overlay of the heparin dimer (pink), and the docked molecules (assorted colors), with respect to arginine 347, 348, and 351 (dark green) A) Tobramycin, B) Neomycin, C) Paromomycin, D) Arbekacin, E) Gentamicin, F) Hyaluronic acid, and G) Plazomicin where the three arginine residues (green) sit with regards to the heparin dimer.

3.4: Conclusions

After multiple rounds of docking it has been determined that aminoglycoside antibiotics could prove to be a useful class of compounds when looking at inhibitors of netrin-1 and its PPI's. The aminoglycoside antibiotics not only sit where the native heparin sits but the binding energies are far lower, in one case over 10 kcal/mol lower than the native ligand.

3.5: References

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Chapter 4: Continuing Work

4.1: Introduction

The docking reported at the end of Chapter 3 showed that aminoglycoside antibiotics were viable PPI inhibitors, based on how they bound to the protein in relation to the native ligand and on the lower binding energies compared to the native ligand. While these criteria are important, they are not the only criteria one must consider when choosing a potential inhibitor candidate. Other things which must be considered include, but are not limited to, whether or not the inhibitor will bind to the desired protein in the correct spot, and whether it will inhibit the PPI. This chapter aims to investigate these criteria, as well as looking at future work that can be done to see if the aminoglycoside antibiotics candidates meet these conditions.

4.2: Aminoglycosides Future Work

One thing to look at when designing an inhibitor is whether a drug or molecule be used in the way that is desired. For example, in this study, can an antibiotic be used as a viable cancer treatment. The answer to this question is yes, as there are currently multiple antibiotics that are used in the treatment of cancer including aminoglycosides.[1] While this is promising, the question of whether or not the antibiotics will interact with the proteins of interest still needs to be answered. For this reason more biotesting needs to be completed. Using either MST, Surface Plasmon Resonance (SPR), or a combination of both; binding, and inhibition studies still need to be completed on the aminoglycoside antibiotics identified during the latest round of docking discussed in Chapter 3. The fact that these known drugs are already approved and purchasable is also good because the synthesis of these stereospecific drugs would be time consuming. A second thing that needs to be done with aminoglycosides is more docking. Using a library of compounds, docking can commence on known aminoglycosides and more candidates can be found to move onto biotesting, increasing the chances of finding successful inhibitors.

4.3: Octet Bio Testing Future Work

Future work on this project will use the Octet system which uses the theory of SPR. The general theory behind SPR is that an analyte is put on a thin metal sheet which sits on a glass dome, then a single wavelength of light is shined into the dome. The light then refracts off of the metal plate due to the surface plasmons and hits a detector; The ligand is then added to the analyte and if there is binding the angle of refraction changes due to the change in the electromagnetic field of the surface plasmons in the metal sheet.[2] This allows

for a real-time look at binding. While that is the basic theory, the Octet system used uses a slightly different method; here the analyte sample is placed in a standard well plate, and the detector is located in a disposable tip that is coated in the target protein.[3] Figure 4.1, shows a better representation of how the Octet system measures binding affinity.[4] As seen in this figure, once binding occurs you can get a real-time shift (blue to orange in this example) to monitor your binding rates and concentrations.

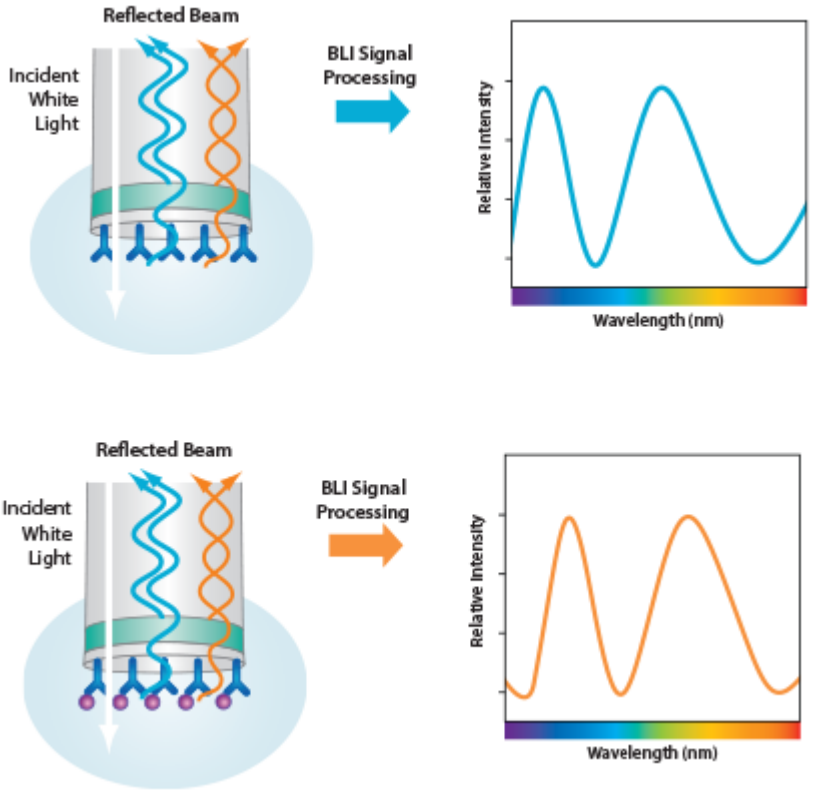

Figure 4.1 Shows how the octet system measures SPR (taken from the Fortebio website)[4].

While MST worked well for biotesting at the beginning of our study, I now have access to an Octet system which uses SPR and should work better for this study as it is a higher throughput, more sensitive instrument that does not require the use of a florescent tag to detect binding.[23] It also allows for samples to be run in well plate, meaning the samples can be loaded faster, more efficiently and replicate data is easier to obtain.

4.4: DCC Future Work

While this project is not yet complete, there is room to expand the use of the methods in other studies. So far, all docking work has looked specifically at the netrin-1 and UNC5 PPI. In the future the same compounds previously docked could be used to look at the PPI of netrin-1 and DCC. The reason to look at DCC next is due to the fact that DCC can bind to netrin-1 in the same hot spot that UNC5 can.[5]

4.5: Conclusions

This study aims to look at identifying inhibiters of PPI's, this is done through the use of computational, synthetic, and biophysical methods. The beginning of the project started with the docking of approximately 90,000 small molecules whose structures are available through various docking libraries. After the completion of the first round of docking the top 0.03% of compounds were once again docked, this time using a longer method looking at more conformations of the small molecules. From this second round of compounds the top five hits were taken. Based on the fact that all of these compounds interacted with the arginine residues in the hotspot, four of these compounds were synthesized, and two of those were able to move onto biological testing using MST and NMR spectroscopy. Based on the data from these tests, it was determined that binding was occurring but not where the docking had predicted, as the hotspot was empty. It was decided that more docking should be completed. Using the fact that netrin-1 binds to heparins in the body, docking of heparin mimics, disaccharides, and approved drugs took place. This docking identified multiple aminoglycoside antibiotics as being potential inhibitors of the netrin-1/UNC5 PPI. Future work will look at testing these antibiotics against netrin-1 to see if these drugs can be repurposed into PPI inhibitors. While the synthesis and biotesting has yet to yield fruitful results, so far docking has shown to be a valuable asset when looking for PPI inhibitors. Not only can more compounds be found that have the potential to inhibit the netrin-1 PPI's, but due to current technology they can be found faster and at a cheaper cost. This may seem like a baseless claim, but so far nearly 100,000 compounds have been explored in this study as potential inhibitors of the netrin-1/UNC5 PPI, and all for the cost of some computing time. Should the aminoglycoside antibiotics inhibit the PPI this will show that using virtual high throughput screening is a viable method and should change how PPI inhibitors are discovered in the future.

4.6: References

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