Assessing HADDOCK’s Protein-Ligand Ensemble Docking Capabilities through Urokinase Inhibitors

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Abstract

Medicinal chemistry is increasingly reliant upon the ability to accurately predict the three-dimensional structures of macromolecular complexes through computational methods\(^1\). One area of particular interest is the prediction of the protein-ligand interactions between small drug molecules and their target proteins. HADDOCK is a macromolecular docking program that uniquely incorporates experimental data about the target protein to drive the docking process\(^1\). Through ensemble docking, I have assessed HADDOCK’s ability to predict the protein-ligand interactions between urokinase protein conformations and eight of its known inhibitors. From these results, HADDOCK is shown to be an effective program for docking urokinase inhibitors when using New 1000 Standard settings. All but two ligands had sampling success of 8/9 or 9/9 and all ligands had the acceptable docked structure within the top 3 scores. Ensemble docking was successful in 6/8 ligand cases with the two failed ligands being of exceptionally small size. If HADDOCK is proven to be a successful docking program across a variety of ligands and proteins, then it could be used to produce more effective drugs with lower production costs and less waste.

Introduction

Proteins and Ligands

Biological proteins are large polymer molecules found throughout the body that facilitate many of its functions. Though they consist of a long linear chain of amino acids, they fold onto themselves resulting in a globular conformation whose shape is dependent upon the order of the amino acids in the chain. Many proteins perform their functions by binding small molecules called ligands to their binding sites, thereby forcing a conformational change to the protein’s structure. This ultimately changes the protein’s function. The broad category of ligands includes the substrates that the protein would normally interact with \textit{in vivo} as well as synthetic drugs that are designed to target the protein. Most drugs work by inhibiting the activity of the receptor protein by binding to its active site\(^2\). Drug development is fundamentally built around the process of finding a small chemical structure that will act as a ligand for a specific protein and will alter the protein’s function.

Conventional Drug Development

At its core, drug development is the identification of a ligand that will bind selectively to a target protein with high affinity\(^3\). High-throughput screening is a method commonly utilized by pharmaceutical companies to achieve this goal. This method involves using robotic automation to screen a large quantity of potential drug molecules by their ability to inhibit a target protein\(^2\). Starting from a fundamental fragment that is expected to bind well to a particular protein, many
similar derivative structures are synthesized as potential drugs\textsuperscript{4}. This process involves a lot of trial and error which leads to a significant amount of wasted money and chemicals while only identifying a few good inhibitor candidates. A rising alternative to the high-throughput screening approach is utilizing computational programs to narrow the field of potential inhibitors of the desired protein and then testing those specific molecules for their inhibitory effects\textsuperscript{4-6}. Calculations that show the molecular conformation with the lowest energy, electrostatic interactions, and docking simulations are used to reduce the number of compounds that need to be synthesized and tested for their drug efficacy\textsuperscript{2}. Focusing limited medicinal chemistry resources on computationally identifying ligands that could act as drugs for target proteins associated with disease is a significantly more efficient way to pursue drug development than a high-throughput screening approach\textsuperscript{4}. Because of this, the continuous improvement of medicinal drug development is heavily dependent upon the ability of a computer program to accurately predict how well a ligand would bind to a specific protein\textsuperscript{3,4}. If a program can accurately model how potential ligands will bind to a target, then this information could be used to develop effective drugs that bind and inhibit the target protein.

**Macromolecular Docking**

Predictive computational programs rely on a process known as macromolecular docking, or simply docking, to identify the three-dimensional structures and intermolecular interactions between two molecules as they would naturally appear in a biological system. This process can be applied to protein-protein interactions, protein-nucleotide interactions, and the protein-ligand interactions that I have focused on for this project\textsuperscript{1,7}. The protocols used to implement docking consist of two major components: a search algorithm and a scoring function\textsuperscript{8}. The search algorithm determines the three-dimensional shapes of the ligand and the protein as they are bound to each other\textsuperscript{8}. The scoring function of the docking process assigns a numeric score to a specific three-dimensional protein-ligand structure that is meant to indicate the affinity between the two molecules. This scoring function is used to rank the small molecules according to their ability to bind to the protein\textsuperscript{8}.

Unfortunately, most docking programs are limited because they must sacrifice modeling realistic protein flexibility for computational speed\textsuperscript{9}. Most docking programs can accurately predict the small ligand’s pose by incorporating complete ligand flexibility into the docking protocol\textsuperscript{8}. However, modeling accurate protein flexibility has proven to be much more difficult because modeling the gargantuan number of potential protein conformations is computationally demanding\textsuperscript{8,10}. Beyond predicting the flexibility of the ligand and the protein individually, a program must also calculate how the protein adapts to accommodate the input ligand when the two structures are introduced to each other. Large ligand-induced changes to the protein conformation can have disastrous effects on the docking performance and must be seriously considered in a docking protocol\textsuperscript{11}.

Because the shape of the bound protein is difficult to predict, many docking programs tend to use a snapshot of the protein structure and make the assumption that it remains rigid
throughout the entirety of the ligand docking process\textsuperscript{8,9}. Within this assumption, the protein is treated as an inflexible “lock” implying that it should be relatively simple to find a ligand “key” that fits complementarily into the protein\textsuperscript{12}. This model of docking fails to produce accurate results about the affinity between a ligand and the protein because flexibility and the positions of particular charged atoms are inherent aspects of the protein’s ability to bind a ligand. Because the protein slightly refolds when a ligand is removed or replaced with a different ligand, the programs that utilize this kind of model are attempting to force a “key” into a protein “lock” that is constantly altering its shape. The “lock-and-key” analogy is an unrealistically simplified model that, despite its shortcomings, has been implemented in pharmaceutical applications because it allows quick screening of large chemical libraries to be done more cheaply.

**HADDOCK-Overview**

HADDOCK (High Ambiguity Data-driven Docking) is a macromolecular docking program that has demonstrated an ability to bypass the inaccuracies caused by the “lock-and-key” model by incorporating limited protein flexibility, thereby allowing the protein to adapt to accommodate different ligands or adapt different input protein conformations to fit the same ligand\textsuperscript{13}. HADDOCK only accounts for the flexibility of the ligand and the residues of the protein that are at the binding interface\textsuperscript{13}. This limited flexibility is attained by the unique way that HADDOCK introduces the ligand to the protein in the docking protocol. The user inputs external information in the form of ambiguous interaction restraints (AIRs), which are used to indicate which residues of the protein are actively involved with binding the ligand\textsuperscript{1,7,14}. By only considering the flexibility of a relatively small area of the protein-ligand complex, HADDOCK provides significant information about the molecular binding and reduces the computational complexity by limiting the binding simulation to a specific part of the protein.

The use of HADDOCK begins with the creation of a protein and a ligand .pdb file (Figure 1). I obtained these by downloading a crystal structure .pdb file from the Protein Data Bank (PDB) and separating it into one protein file and one ligand file. These two input files are submitted to the HADDOCK webserver (http://milou.science.uu.nl/services/HADDOCK2.2/) along with the AIRs files and the docking parameters. HADDOCK then connects the ligand file to the protein file according to the restraints indicated in the AIRs files. Following this, HADDOCK implements a three-stage docking process (This is explicitly discussed in the Methods and Procedure section). Each stage simulates varying degrees of protein flexibility which attempts to model how a real protein would fold and interact with the ligand. This protocol is repeated 200 times and generates 200 structures that have the ligand docked into the protein. Some of these structures may correctly mimic an \textit{in vivo} interaction and are considered acceptable while others may be incorrectly docked. In the example shown in Figure 1, the acceptable structures will all be similar to the correct structure shown with the double ring of ligand 239 pointing into the 4FUC urokinase protein. The incorrect structures will be more variable, the figure shows an example of an incorrect docking, with the ligand rotated 180° from the correct structure with the double ring of the ligand pointing out of the protein.
Figure 1. Summary of the HADDOCK docking process. A ligand and a protein .pdb file are created and submitted to the HADDOCK webserver. Submitted AIRs files determine how the two files are brought together. The webserver runs a 3-stage docking protocol 200 times and generates 200 predicted protein-ligand complex structures. Some of these structures have the ligand correctly docked, others do not. These structures are then assigned scores and go through a grouping process called clustering.

Each of the 200 structures is assigned a score by HADDOCK, referred to as the HADDOCK score. HADDOCK then divides the 200 structures according to their structural similarity through a process called clustering. The proteins are overlaid, and the positions of the ligands are compared. Ligands that deviate from each other by less than 2 Å are grouped into a cluster. Following this, each structure is manually measured in a program called ProFit to obtain an i-RMSD (ligand-Root Mean Square Deviation) value. This is a measurement of how different the ligand pose predicted by HADDOCK is from the original crystal structure from the PDB. Finally, the i-RMSD values are compared to the HADDOCK scores. The goal is for the cluster with the best i-RMSD score to also have the best HADDOCK score thereby indicating that HADDOCK is attaching the best score to the most correct protein-ligand structure.
HADDOCK-AIRs

As mentioned before, AIRs are what dictate HADDOCK’s unique docking process. AIRs indicate which protein residues are active and involved in the interactions with the docked molecule\(^7\). These residues are influenced by varying degrees of flexibility throughout the docking protocol. This allows HADDOCK to dock the ligand into the protein with a highly localized consideration for the flexibility of the protein.

Prior information regarding the location of the protein’s binding site is necessary to create AIRs. Methods of obtaining this information include using perturbation data from NMR titration experiments or x-ray crystallography\(^7,14\). The area of a protein that interacts with a ligand can be found and identified as the protein’s ligand-binding site. This information is stored by researchers in the PDB. Crystal structures that include desirable target proteins and their known substrates can be downloaded from this database. If a protein has been identified as a possible drug target, the location of the protein’s binding site is valuable information. A drug that is meant to inhibit the protein will typically need to target the same binding site that the \textit{in vivo} substrate binds to. As such, AIRs need only to include the known binding site of the protein and need not include other ligand-binding or allosteric sites.

For this experiment I have used a set of nine urokinase protein-ligand crystal structures. The ligands are known urokinase inhibitors that bind to the same binding site (Figure 2). They are a series of related compounds with a naphthyl-amidine functional group and variously sized additional functional groups. The published crystal structures that contain these ligands are used as validation for HADDOCK’s docking method. For each crystal structure, I created a list of protein residues that are within 3.9 Å of the ligand. I chose the most expansive list of protein residues with the most residues in common with other lists. This has provided a reliable set of residues that are consistently part of the binding site to be conveyed through the AIRs.
Figure 2. Superimposition of the eight inhibitor ligands in the urokinase binding site. 1UP (Blue), 2UP (Green), 675 (Yellow), 4UP (Purple), 239 (Cyan), 6UP (Orange), 7UP (Gray), and 132 (Red) are displayed over each other using PyMOL. All eight ligands share a binding site and share many contact residues. The set of protein residues that are used in AIRs are shown in Orange.

HADDOCK-Clustering, Scoring, and i-RMSD

Following HADDOCK’s docking protocol, the output structures that have predicted how the ligand would dock into the protein structure are analyzed (Figure 3). To make the 200 output structures more manageable, HADDOCK groups structures with similar poses into clusters. The protein-ligand structures that are within 2 Å of each other are grouped into a single cluster. Figure 4 shows the visual differences between two clusters generated by a single HADDOCK docking of ligand 132 into the non-native 4FUC urokinase protein conformation. Both clusters are overlaid on the crystal structure shown in green. The cluster on the left has an i-RMSD value below 2 Å, classifying it as an acceptable structure, and it closely lines up with the crystal structure. The nitrogen atom (Blue) and the oxygen atom (Red) at the bottom of the ligand are positioned almost identically. The unacceptable docked cluster on the right has an i-RMSD value above 2 Å, is rotated 180° over two axes, and is effectively removed from the binding pocket. In this instance, the acceptable cluster also has a HADDOCK score that is much more negative than the unacceptable cluster. This example shows HADDOCK accurately assigning the better score to the cluster with the more accurate structure.
Figure 3. Summary of the quantitative analysis of HADDOCK output structures. The 200 structures are separated into 1-10 clusters according to their similarity. Each structure is assigned a HADDOCK score and an i-RMSD value is measured. The average of the HADDOCK scores of the four best scoring structures of each cluster is taken to produce one HADDOCK score per cluster. The i-RMSD values for these four structures are averaged to produce one i-RMSD value for each cluster. The one HADDOCK score and the one i-RMSD value are compared to generate a graph for each docking (Figure 5).

Figure 4. Example of an acceptable (left) and an unacceptable (right) cluster. Both examples show non-native docked ligand 132 (White) in the protein 4FUC overlaid on the crystal structure containing ligand 132 (Green). HADDOCK has assigned the acceptable structure a much better HADDOCK score.
The HADDOCK score is a measurement of the change in stability from an unbound protein to a ligand-bond protein. It is a measurement of the decrease in free energy, so a more negative value corresponds with a more energetically favorable protein-ligand structure. For each output structure that it produces, HADDOCK calculates the van der Waals energy ($E_{vdw}$), the electrostatic energy ($E_{elec}$), the desolvation energy ($E_{desolv}$), and docking restraints energy ($E_{air}$). The HADDOCK score for the structure is a weighted sum of these four values$^{13}$. The buried surface area (BSA) is a measurement of the exposed surface area of both molecules minus the exposed surface area of the complex of the two molecules. The BSA value only affects the it0 and it1 stages of docking and is subtracted from the rest of the HADDOCK score.

New1000 Standard settings:

(it0) $1.0E_{vdw} + 1.0E_{elec} + 1.0E_{desolv} + 0.01E_{air} - 0.01BSA = HADDOCK$ score

(it1) $1.0E_{vdw} + 1.0E_{elec} + 1.0E_{desolv} + 0.1E_{air} - 0.01BSA = HADDOCK$ score

(itw) $1.0E_{vdw} + 0.1E_{elec} + 1.0E_{desolv} + 0.1E_{air} - 0.0BSA = HADDOCK$ score

New1000 Buried Site settings:

(it0) $0.0E_{vdw} + 1.0E_{elec} + 1.0E_{desolv} + 0.01E_{air} - 0.01BSA = HADDOCK$ score

(it1) $1.0E_{vdw} + 1.0E_{elec} + 1.0E_{desolv} + 0.1E_{air} - 0.01BSA = HADDOCK$ score

(itw) $1.0E_{vdw} + 0.1E_{elec} + 1.0E_{desolv} + 0.1E_{air} - 0.0BSA = HADDOCK$ score

After a HADDOCK score is calculated for each structure, they are ranked according to this score within each cluster. The four best-scoring structures in each cluster are used to represent the entirety of the cluster. The average of these four HADDOCK scores is used to generate the single HADDOCK score used to represent the entire cluster. i-RMSD values are found by measuring the difference between the output structure’s ligand pose and the crystal structure’s ligand position. This measurement is used to determine if the ligand has been correctly docked into the protein structure by HADDOCK. Any i-RMSD value under 2 Å is a small enough difference to be considered an acceptable docking that would accurately replicate how the ligand binds to the protein in the crystal structure. The i-RMSD values for each of the four best scored structures in each cluster are averaged to generate a single i-RMSD for the cluster.

From the single HADDOCK score and i-RMSD value for each cluster, a graph like the one shown in Figure 5 can be created. This figure combines two HADDOCK runs in which ligand 4UP was docked into the urokinase structure 4FUC using different docking parameters. The parameters for New 1000 Standard settings and for New 1000 Buried Site settings are detailed in the Methods and Procedure section. Each data point represents a single cluster from the HADDOCK output and the red line shows the cutoff for acceptable structures at 2Å i-RMSD. An increasing linear trend is ideal to show that correct dockings are assigned a better HADDOCK score than nearly-correct or incorrect dockings. In Figure 5 the New 1000 Standard settings show a good linear trend in which the most correct cluster is scored the best by HADDOCK. The New 1000 Buried Site settings show no trend. The two data points at ~1 Å and
~2 Å i-RMSD have roughly the same HADDOCK score even though one is acceptable, and one is not. Additionally, the two points at ~8.5 Å i-RMSD indicate that two incorrectly docked clusters were scored very well.

**Figure 5.** Graphical representation of a HADDOCK run’s compiled data. Each data point is representative of an entire cluster from the output of a HADDOCK run. The data from a run using the New1000 Standard settings and the data from a run using the New 1000 Buried Site settings with the same input protein and ligand are combined into one graph. The red line shows the cutoff for acceptable structures at 2 Å i-RMSD.

One of these graphs was produced for every combination of the eight urokinase inhibitors docked into nine urokinase protein structures. From these 72 graphs, presented in the Supplementary Information section, I produced the rest of the data that is presented.

**Ensemble Docking**

In a benchmark exercise, the Grinstead lab has docked 78 native complexes using 10 Å AIRs with top 1 scoring success of 77% and sampling success of 92%. Additionally, 71 non-native conformations corresponding to 56 native complexes were docked using 10 Å AIRs with top 1 scoring success of 66% and 83% sampling success. The benchmark exercise utilized a protein and ligand set that contained nearly all of the molecules that constitute the Astex diverse set. For reference, the fairest comparison is GOLD’s docking performance with the complete Astex Diverse Set. Using this set, the GOLD docking program achieved an 80% top 1 scoring success and a 91% sampling success for native dockings. GOLD also achieved a 61% top 1 scoring success and a 72% sampling success for non-native dockings. Using this measure of success, HADDOCK and GOLD have comparable success rates with a similar and significant decrease in success when docking non-native complexes.

In an attempt to increase HADDOCK’s success with non-native dockings, I employed a method known as ensemble docking. Ensemble docking involves using multiple discrete conformations of the same protein to account for some of the protein’s flexibility without
needing to perform complete protein flexibility calculations. Multiple conformations of the same protein can be found in the PDB, each bound to a different ligand as a crystal structure. Each conformation has adapted to accommodate its ligand and as a result will have a slightly differently shaped binding site. In this project’s ensemble docking, a single urokinase inhibitor was docked into nine urokinase protein conformations, each one utilizing the localized flexibility defined by AIRs. By combining ensemble docking and AIRs-driven docking methods, more urokinase flexibility is considered while still remaining computationally feasible. The data from these nine dockings was compiled to create a more comprehensive model of how that ligand might bind to the urokinase protein. This process was repeated for each of the other seven urokinase inhibitors in my set to determine if the method could be generally applied.

**Urokinase**

Urokinase-type plasminogen activator (uPA), commonly known as urokinase, is a common target protein for anti-tumor drugs. In vivo, urokinase binds to the membrane-anchored urokinase-type plasminogen activator receptor (uPAR). While bound to uPAR, the urokinase enzyme cleaves plasminogen, converting it into its active plasmin form. In a normal physiological role, the active plasmin breaks down fibrin, a protein meshwork that plays a role in blood clotting. This breakdown of fibrin contributes to the normal process that allows wounds to heal. The uPA-uPAR system participates in the intracellular signaling pathways responsible for cell proliferation, survival, and migration. After being cleaved by uPA, the activated plasmin is also capable of initiating a proteolytic cascade to degrade the extracellular matrix (ECM) and ultimately lead to metastasis. Because of its roles in fibrin breakdown in blood clots and metastasis, understanding the uPA-uPAR system is beneficial to cancer research and medication development.

One effective way to inhibit the urokinase protein is to develop a drug that binds competitively to the substrate binding site. By introducing a molecule that binds to urokinase at its catalytic domain, the biological substrate cannot be activated by urokinase. Pharmaceutical companies are trying to develop a specific urokinase inhibitor with varying success. A more effective way to produce a drug to target urokinase would be to first understand how well a chemical structure would bind to the protein. If a protein-ligand docking program could accurately predict this information, it would pave the way for the production of more effective and more specific urokinase-targeting medications. If HADDOCK ensemble docking can be used to effectively predict the protein-ligand interactions between the urokinase protein and its known inhibitors, it could be a powerful tool in cancer drug development.
Figure 6. a) Physiological role of urokinase (uPA) in activating plasminogen leading to the breakdown of the fibrin meshwork\textsuperscript{16}. b) Broader role of urokinase and uPAR trimer in ECM degradation, metastasis, and intracellular signaling cascades\textsuperscript{16}. 
Methods and Procedure

HADDOCK Files

Urokinase protein structures and known urokinase inhibitor ligand structures were obtained as .pdb files from crystal structures in the RCSB Protein Data Bank. The specific urokinase crystal structures were chosen primarily to correspond with the Community Structure Activity Resource (CSAR) 2012 benchmark exercise. The crystal structures containing the 1GJ7 and 1OWE protein conformations were chosen because of their past use in the Grinstead lab. A full list of the urokinase protein conformations and the corresponding native ligands is shown in Table 1.

Table 1. Urokinase proteins and native ligands denoted by their PDB codes.

<table>
<thead>
<tr>
<th>Urokinase Conformation</th>
<th>Native Ligand</th>
<th>Native Ligand Structure</th>
<th>Affinity (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4FU7</td>
<td>1UP</td>
<td><img src="image1" alt="Image" /></td>
<td>0.637</td>
</tr>
<tr>
<td>4FU8</td>
<td>2UP</td>
<td><img src="image2" alt="Image" /></td>
<td>5.91</td>
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<tr>
<td>4FU9</td>
<td>675</td>
<td><img src="image3" alt="Image" /></td>
<td>0.628</td>
</tr>
<tr>
<td>Compound</td>
<td>Value</td>
<td>Molecular Structure</td>
<td></td>
</tr>
<tr>
<td>----------</td>
<td>-------</td>
<td>---------------------</td>
<td></td>
</tr>
<tr>
<td>4FUB</td>
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<td>4FUC</td>
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<td>0.0588</td>
<td><img src="image4" alt="Molecular Structure" /></td>
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</tr>
<tr>
<td>1GJ7</td>
<td>0.013</td>
<td><img src="image5" alt="Molecular Structure" /></td>
<td></td>
</tr>
<tr>
<td>1OWE</td>
<td>0.628</td>
<td><img src="image6" alt="Molecular Structure" /></td>
<td></td>
</tr>
</tbody>
</table>
Restraints Generation

3.9 Å contact residues were calculated for each crystal structure (Table 2). The AIRs files utilized in each HADDOCK run were created using the 3.9 Å contact residues from the 4FUC-239 crystal structure. This set of residues was chosen to be the consensus set because it has the most expansive set of residues and has significant overlap with other residue sets. The set from the 1GJ7-132 crystal structure is more expansive but was added late to the project and was not yet considered. The set of residues used in all AIRs files is shown visually as part of the 4FUC urokinase protein structure in Figure 7.

Table 2. Lists of 3.9 Å contact residues for each ligand and residues used in AIRs. Ligand 675 is the native ligand in two crystal structures.

<table>
<thead>
<tr>
<th>Crystal Structure</th>
<th>3.9 Å contact residues</th>
</tr>
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<tbody>
<tr>
<td>4FU7-1UP</td>
<td>192, 193, 194, 195, 198, 216, 217, 218, 219, 220, 221, 222, 229</td>
</tr>
<tr>
<td>4FU8-2UP</td>
<td>192, 193, 195, 198, 217, 218, 219, 221, 222, 229</td>
</tr>
<tr>
<td>4FU9-675</td>
<td>46, 94, 192, 193, 195, 198, 218, 219, 221, 222, 229</td>
</tr>
<tr>
<td>4FUB-4UP</td>
<td>46, 94, 192, 193, 195, 198, 218, 219, 221, 222, 229</td>
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<tr>
<td>4FUC-239</td>
<td>46, 50, 94, 192, 193, 195, 198, 217, 218, 219, 221, 222, 229</td>
</tr>
<tr>
<td>4FUD-6UP</td>
<td>192, 193, 195, 198, 216, 218, 219, 221, 222, 229</td>
</tr>
<tr>
<td>4FUE-7UP</td>
<td>46, 50, 192, 193, 195, 198, 218, 219, 221, 222, 229</td>
</tr>
<tr>
<td>1GJ7-132</td>
<td>31, 46, 47, 192, 193, 194, 195, 198, 216, 217, 218, 219, 221, 222, 229, 230</td>
</tr>
<tr>
<td>1OWE-675</td>
<td>46, 94, 192, 193, 195, 198, 216, 218, 221, 222, 229</td>
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<tr>
<td>In AIR Files</td>
<td>46, 50, 94, 192, 193, 195, 198, 217, 218, 219, 221, 222, 229</td>
</tr>
</tbody>
</table>

Figure 7. Urokinase protein residues used in AIRs. The 4FUC-239 contact residues used in HADDOCK runs through AIRs are shown in red.
HADDOCK Protocol

The HADDOCK protocol is shown graphically in Figure 8. The protein.pdb file, ligand.pdb file, and AIRs were prepared and submitted to HADDOCK. The protein and ligand structures were held at 50 Å from each other to eliminate interactions between the molecules. The ligand was introduced to a rigid body model of the protein according to the protein residues indicated in the AIRs. From this rigid body docking model, 2000 protein-ligand complexes were generated. These complexes were subjected to a two-step, semi-flexible protein simulation from which the best 200 complexes were selected. At the final stage, these 200 complexes were surrounded by solvent (water). Following water refinement, the 200 structures were clustered according to their similarity and analyzed.

**HADDOCK New1000 Standard Settings Protocol**

Crystal Structure (.pdb file)

Ligand (.pdb file)  Protein (.pdb file)

Positions of protein and ligand at 50 Å away from each other

**AIRs**

Final Refinement in explicit solvent (water) 200 complexes

Rigid Body energy minimization 2000 complexes generated

Semi-Flexible simulated annealing
1) Side chains
2) Side chains + backbone
200 complexes

Clustering and Analysis
Scoring of clusters according to intermolecular energy (Evdw, Eelec, AIR, ...)

**HADDOCK New1000 Buried Site Settings Protocol**

Modifications to the Rigid Body docking stage only:
- Reduced intermolecular interaction energy (1% of Standard)
- Reduced van der Waals energy component of scoring to zero

Figure 8. HADDOCK docking protocol with Standard and Buried Site settings. Each combination of protein and ligand was docked using both docking protocols.

* Ambiguous Interaction Restraints (AIRs) direct HADDOCK to dock the ligand into the protein’s binding site. AIRs guide the interactions at each stage in the docking process.
Parameters Utilized

Each protein-ligand combination was docked according to two sets of docking parameters: New 1000 Standard settings and New 1000 Buried Site settings. The docking protocol for each was identical apart from the parameters listed below. The Buried Site settings reduced the intermolecular interaction energy to just 1% of the Standard settings. Additionally, the van der Waals energy component of the HADDOCK score was removed entirely. These changes were made to allow ligands in Buried Site dockings to access and bind to spots of the protein that would normally be inaccessible. This was intended to increase the likelihood that an acceptable protein-ligand complex was predicted by allowing the ligand to assume more poses and access parts of the binding site that are otherwise sterically blocked during the rigid body stage of docking.

New 1000 Standard Settings- Obtained by altering the default settings as follows:

- **Distance restraints**
  - Change “Remove non-polar hydrogens” to false

- **Parameters for clustering**
  - Change “Clustering method” to RMSD
  - Change “RMSD Cutoff for clustering” to 2.0

- **Restraints energy constants**
  - **Energy constants for unambiguous restraints**
    - Change “First iteration” to 1
  - **Energy constants for ambiguous restraints**
    - Change “Last iteration” to 0

- **Scoring parameters**
  - Change “Evdw 1” to 1.0
  - Change “Eelec 3” to 0.1

- **Advanced sampling parameters**
  - Change “number of MD steps for rigid body high temperature TAD” to 0
  - Change “number of MD steps during first rigid body cooling stage” to 0

New 1000 Buried Site Settings- Obtained by altering the default settings as follows:

- **Distance restraints**
  - Change “Remove non-polar hydrogens” to false

- **Parameters for clustering**
  - Change “Clustering method” to RMSD
  - Change “RMSD Cutoff for clustering” to 2.0

- **Restraints energy constants**
  - **Energy constants for unambiguous restraints**
    - Change “First iteration” to 1
  - **Energy constants for ambiguous restraints**
- Change “Last iteration” to 0
- **Energy and interaction parameters**
  - Change “Scaling of intermolecular interactions for rigid body EM” to 0.01
- **Scoring parameters**
  - Change “Evdw 1” to 0.0
- **Advanced sampling parameters**
  - Change “number of MD steps for rigid body high temperature TAD” to 0
  - Change “number of MD steps during first rigid body cooling stage” to 0

**Cluster Analysis**

Following the HADDOCK docking protocol, the output structures were clustered according to their similarity and analyzed. The output structures within 2 Å i-RMSD of each other were grouped into a single cluster. All structures were assigned a score by HADDOCK equal to the weighted sum of the van der Waals energy (E_{vdw}), the electrostatic energy (E_{elec}), the desolvation energy (E_{desolv}), and the distance restraints energy (E_{air}) of the docked structure. The BSA value is subtracted from the rest of the score.

New1000 Standard settings:

- (it0) $1.0E_{vdw} + 1.0E_{elec} + 1.0E_{desolv} + 0.01E_{air} - 0.01BSA = HADDOCK\ score$
- (it1) $1.0E_{vdw} + 1.0E_{elec} + 1.0E_{desolv} + 0.1E_{air} - 0.01BSA = HADDOCK\ score$
- (itw) $1.0E_{vdw} + 0.1E_{elec} + 1.0E_{desolv} + 0.1E_{air} - 0.0BSA = HADDOCK\ score$

New1000 Buried Site settings:

- (it0) $0.0E_{vdw} + 1.0E_{elec} + 1.0E_{desolv} + 0.01E_{air} - 0.01BSA = HADDOCK\ score$
- (it1) $1.0E_{vdw} + 1.0E_{elec} + 1.0E_{desolv} + 0.1E_{air} - 0.01BSA = HADDOCK\ score$
- (itw) $1.0E_{vdw} + 0.1E_{elec} + 1.0E_{desolv} + 0.1E_{air} - 0.0BSA = HADDOCK\ score$

The structures with the four best HADDOCK scores were averaged to generate a single HADDOCK score for each cluster.

Using ProFit, the ligand-root mean square deviation (i-RMSD) of each output structure was measured. An i-RMSD under 2 Å indicated a structure had a ligand pose sufficiently similar to the crystal structure and therefore was acceptable. This standard of acceptability is the most commonly used performance indicator for docking programs\(^1^1\). The i-RMSD value of the structures with the four best HADDOCK scores were averaged to generate a single i-RMSD value for each cluster. A cluster with an averaged i-RMSD value under 2 Å was considered to be acceptable.
Results

Compiled Data

From the graphs presented in the Supplemental Information section, the success rates in Tables 3-8 were determined and compiled. In Tables 3-5, each row shows the number of urokinase protein conformations in which a particular urokinase ligand was successful. Each ligand was docked into nine urokinase conformations (4FU7, 4FU8, 4FU9, 4FUB, 4FUC, 4FUD, 4FUE, 1OWE, 1GJ7). An acceptable cluster is considered to be one with an i-RMSD value below 2 Å. Sampling success is defined as the HADDOCK run producing at least one acceptable output cluster, regardless of its score. Top 1 scoring success is defined as the output cluster with the best HADDOCK score also being an acceptable cluster. Top 2 scoring success is defined as at least one of the best two scoring clusters also being an acceptable cluster. Top 3 scoring success is defined as at least one of the best three scoring clusters also being an acceptable cluster. Each cell is /9 and indicates the number of protein conformations in which each ligand had success when it was docked. e.g. In Table 3, ligand 6UP has a sampling success of 9/9. For all nine protein conformations that 6UP was docked into, each docking produced at least one output cluster that was acceptable.

Table 3. Compiled Data of New 1000 Standard Settings

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Sampling Success</th>
<th>Top 1 Scoring Success</th>
<th>Top 2 Scoring Success</th>
<th>Top 3 Scoring Success</th>
</tr>
</thead>
<tbody>
<tr>
<td>1UP</td>
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<td>9/9</td>
<td>9/9</td>
<td>9/9</td>
</tr>
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Table 4. Compiled Data of New 1000 Buried Site Settings

<table>
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</thead>
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<td>239</td>
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Table 5. Compiled Data of Combined New 1000 Standard and Buried Site Settings

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<th>Top 1 Scoring Success</th>
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</table>

In tables 6-8, each row shows the number of ligands that each protein could successfully accommodate. Each protein was docked with eight urokinase inhibitors (1UP, 2UP, 675, 4UP, 239, 6UP, 7UP, 132). The same measurements of success are used as before. Each cell is /8 and indicate how many ligands each protein conformation had success with when docking it.
### Table 6. Compiled Data of New 1000 Standard Settings

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<th>Protein</th>
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### Table 7. Compiled Data of New 1000 Buried Site Settings

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</table>

### Table 8. Compiled Data of Combined New 1000 Standard and Buried Site Settings

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<th>Protein</th>
<th>Sampling Success</th>
<th>Top 1 Scoring Success</th>
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<td>4/8</td>
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</table>
It is valuable to note that the Top 3 Scoring Success column for the Standard settings data exactly matches the Sampling Success column in both the ligand and the protein tables (Tables 3 and 6). This indicates that for every docking run that utilized the Standard settings and produced at least one acceptable cluster, an acceptable cluster was always found within the top three HADDOCK scores.

**Ensemble Docking**

As discussed before, ensemble docking involves docking a single ligand into many protein conformations and comparing the best scores from each docking to get a more accurate representation for how a ligand would bind. Ideally, the protein conformation that scores the best would also be an acceptable structure. Figure 9 shows the top scoring cluster from each protein-ligand combination with each set of docking parameters. If the cell is green, the cluster that received the top score was acceptable. If the cell is red, the top scoring cluster was unacceptable. The protein conformation that scored the best for each ligand is outlined in orange. If the protein conformation that scored the best for each ligand is also an acceptable structure, then the ensemble docking was successful for that ligand. Ensemble docking success was 6/8 using the data from Standard settings, 3/8 using data from Buried Site settings, and 4/8 using the combined data from the two settings. The 1GJ7 urokinase conformation consistently produced the best score, regardless of the parameters that were used in the docking. However, the second-best scoring conformation was more variable implying that some unique characteristic of 1GJ7 allowed it to score significantly better than the other urokinase conformations. The two ligands 2UP and 6UP failed ensemble docking regardless of the parameters used in the docking.

a) New 1000 Standard Settings Data

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b) New 1000 Buried Site Settings Data

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c) Combined New 1000 Standard and Buried Site Data

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</table>

**Figure 9.** Results of ensemble docking. The top score of each protein-ligand docking is listed and colored green if it is acceptable and colored red if it is unacceptable. The best score for each ligand row is outlined in orange. If the orange-outlined cell is also acceptable, the ensemble docking was successful for that ligand. Ensemble docking success is a) 6/8, b) 3/8, c) 4/8

**ir-RMSD vs i-RMSD**

Site root mean square deviation (ir-RMSD) is a calculation used to measure the difference between the binding sites of two protein conformations. It was found using ProFit to measure the RMSD of the protein residues designated by the AIRs as the binding site. Each protein conformation has an ir-RMSD value vs each other protein as shown in Figure 10. The ir-RMSD was set against the best i-RMSD value that was generated by docking the consistent protein conformation’s native ligand into each other protein using both the Standard and Buried Site settings. This was intended to determine whether the difference between two conformations’ binding sites influenced how well one conformation’s native ligand docked into the other protein conformation. A positive linear trend would indicate that docking a ligand into a non-native protein conformation with a significantly different binding site would predict structures that are less similar to the crystal structures. Because there is no such trend in any of the graphs, it can be concluded that the difference in binding site shape does not significantly affect ligand docking.
HADDOCK can dock a ligand to all of the conformations regardless of the initial differences in the site conformation from the native conformation. All graphs resembled each other except for the bottom graph in which the ir-RMSD vs 1GJ7 is noticeably higher than the ir-RMSD values in the other graphs. This indicates that 1GJ7 is the conformation whose binding site is the most different from that of the other urokinase structures.

**Figure 10.** ir-RMSD vs i-RMSD for each urokinase conformation. ir-RMSD was measured with ProFit for each conformation against one consistent conformation for each graph. This value was set against the best i-RMSD value for each docking that combined the protein conformations and the consistent conformation’s native ligand.
Sampling Success of Ligands

Most ligands had 8/9 or 9/9 sampling success when they were docked with Standard settings. Introducing the Buried Site settings tended to hurt sampling success and as such was not considered in the ligand success analyses. The only ligands that did not achieve an 8/9 or 9/9 sampling success were ligands 675 and 132.

Ligand 675 had a Standard setting sampling success of 6/9 and more closely resembles the structures of other ligands in the set. It contains the naphthyl-amidine functional group that is shared across most ligands and is more linear. This may be why its sampling success was only slightly below the 8/9 and 9/9 group.

Ligand 132 had a Standard setting sampling success of 4/9, performing the worst of any ligand in the group. This ligand is large and has a bent shape with free rotation around the C1’-C8 bond. It is also the only ligand without the naphthyl-amidine functional group. It may be these structural differences that caused its 4/9 sampling success.

Scoring Success of Ligands

The Standard settings gave the best scoring success for all but two ligands, 2UP and 6UP. Top 1 scoring success and top 2 scoring success were improved for these ligands by using the Buried Site settings and the combined data of the Standard and Buried Site settings. Top 3 scoring success, however, was slightly better when using Standard settings. With Standard settings the top 3 scoring success column matches the sampling success column. Ligands 2UP and 6UP were the only ligands that did not have an acceptable cluster until the 3rd best score whereas all other ligands had an acceptable cluster on the 1st or 2nd best score. These two ligands also consistently failed ensemble docking (Figure 9).

These two ligands are clearly the smallest of the set. 2UP is composed of just the base naphthyl-amidine group and 6UP only has one additional nitrogen group. The small size of the ligands may be affecting HADDOCK’s ability to score them properly.
Negative Control

To determine HADDOCK’s ability to distinguish binding inhibitors from molecules that will not bind to urokinase, I have submitted two “negative control” molecules, uPA 33 and uPA 45. These molecules were identified as ligands with >100μM affinity for urokinase in the CSAR 2012 benchmark exercise. I have docked these non-binding ligands into each of the nine protein conformations to determine if HADDOCK will score them poorly.

For comparison, the other ligands in my set had affinities that ranged from 0.013 μM (132) to 5.91 μM (2UP). One would expect the non-binding ligands to score worse than any in the inhibitor ligand set. Figure 11 shows this analysis graphically by plotting the average of the HADDOCK scores for all of the clusters produced by each docking. In the Buried Site setting dockings, the non-binding ligands did not score much worse than the inhibitor ligands. Sometimes they even scored better. In the Standard setting dockings, the non-binding ligands tended to score worse than the inhibitor ligands. Though the difference does not appear to be very large in Figure 11, it is important to remember that the range in affinities of the binding ligands extends across several orders of magnitude. As such, the difference between the binding and non-binding ligands’ abilities to bind to urokinase is less stark. From this analysis it appears that HADDOCK using Standard settings could reliably assign non-binding ligands worse scores, though improvements to the scoring function could improve separation between the ligand scores.

The similarity in scores may be due to the relatively small set of residues used in AIRs. Because the set only extended to 3.9 Å contact residues, the ligand was not allowed to stray far from the binding pocket. If the AIRs included residues up to 10 Å from the ligand, the non-binding ligands may have been docked to areas outside of the binding site, leading to less energetically favorable scores.
Figure 11. HADDOCK scoring of non-docking ligands compared to known inhibitors. The average of the cluster scores for each docking were compiled. The triangular data points represent the non-binding ligands uPA33 and uPA45.

Discussion

Identifying the Correct Cluster

While consistent top 1 scoring success is obviously ideal, it isn’t the only determinant of HADDOCK’s usefulness. When Standard settings were used, top 3 scoring success equals sampling success for each of the proteins and the ligands. This indicates that if a ligand was docked into a protein and produced at least one acceptable cluster, one of the acceptable clusters scored within the top 3 scores in every instance. The same is true for the proteins, indicating that if a protein was able to accept a ligand and produced at least one acceptable cluster, one of these clusters was within the top 3 scores. Because an acceptable cluster, when it exists, can always be found within the top 3 scores, the number protein-ligand complexes that could model an in vivo
interaction are significantly narrowed. Supplemental, outside information can be used along with the HADDOCK results to determine which of these three clusters correctly models the protein-ligand binding.

One effective tool to determine this is a visual comparison of protein-ligand structures with PyMOL. Each output structure predicted by HADDOCK has a .pdb file associated with it. If the protein is a highly desired drug target, it is likely that it has been crystalized with some sort of ligand and uploaded to the PDB. This crystal structure also has a .pdb file associated with it. By opening the HADDOCK output structures and the crystal structure together in PyMOL, it is apparent whether the ligand in the crystal structure and the docked ligand are bound at the same spot in the protein’s binding site. HADDOCK clusters with ligands docked at an energetically favorable place on the periphery of the binding site that was designated by AIRs may have good HADDOCK scores but be unacceptable clusters. These clusters could potentially be dismissed through visual methods if they do not bind to the same site as the crystal structure’s ligand.

In addition to the visual analysis, other scores calculated by HADDOCK can also be used to identify the correct clusters. For example, the $E_{\text{air}}$ values for a cluster indicate whether the ligand is in contact with at least one residue from the AIRs files. When the distance restraints are satisfied the $E_{\text{air}}$ value is zero. A value greater than zero shows that the ligand is not in contact with the binding site that has been specified. Analysis of the scores that are distinct from the overall HADDOCK score can be used to help to identify which cluster from a small group has the more favorable binding.

**Docked Ligand Charges**

Urokinase inhibitors possessing the naphthyl-amidine functional group bind to the protein by forming hydrogen bonds and an ionic bond with aspartic acid 192 (ASP 192), serine 193 (SER 193), and glycine 221 (GLY 221) as shown by the interaction below. Successful dockings consistently formed these bonds between the ligand and protein.
One factor that could significantly influence dockings is the charges of atoms within a ligand molecule\(^9\). Prior to docking, the ligand structure is submitted to the PRODRG server which produces an energy-minimized three-dimensional structure to be used in the docking. If the PRODRG output structure assigns a protonation state or a charge to an atom that does not realistically reflect that of the ligand \textit{in vivo}, the docking’s accuracy could be affected.

In this set of ligands, the charges assigned by PRODRG were very consistent. Every naphthyl-amidine functional group and ligand 132’s similar functional group had the same charge distribution on the binding atoms. Ligand 2UP is shown below as a model for the naphthyl-amidine functional groups. Each ligand was docked with two hydrogen atoms on each of the nitrogen atoms (NAB and NAA). The nitrogen atoms had very low partial charges ($\delta \approx 0.1$) with the carbon atom between them (CAJ) possessing a slightly elevated charge ($\delta \approx 0.3$). This indicates a transient positive charge throughout these three atoms. Because this functional group is consistently used in binding to the protein structure, the charges or protonation states on these atoms are necessary to form the interactions between the ligand and the residues ASP 192, SER 193, and GLY 221.

```
 Additional Cofactors
```

X-ray crystallography is the primary method used to create the crystal structures in the PDB and identify the residues at the protein’s binding site. A protein must first be experimentally crystallized for it to be analyzed through x-ray crystallography. To maintain the stability and accurate shape of the molecule throughout this process, the protein must have a ligand in its binding site and is subjected to unique environmental conditions. These conditions rarely reflect the environment that the protein is exposed to \textit{in vivo} and can result in the crystallized protein being bound to small ions or molecules that are not ligands. The protein-ligand structure and the extra molecules are logged digitally as a crystal structure in the PDB. These are the crystal structures that were used as the starting files in this study.

It is difficult to know what to do with these extra molecules. Non-ligand cofactors in or near the ligand-binding site could influence how a ligand is docked into a protein structure by creating forces that are not present with \textit{in vivo} conditions. However, the cofactors may also be necessary to maintain the structure of the protein as it is subjected to docking conditions by a program that cannot replicate the complexity of \textit{in vivo} environments. In this project, only the inhibitor ligand molecules were separated into their own .pdb file. All other cofactors were left in the crystal structure and became part of the protein .pdb file. A list of the additional cofactors found in the binding site of each protein conformation is provided in Table 9. Given more time, the project would be replicated using protein .pdb files that have had all cofactors removed to determine if the extra molecules influenced HADDOCK’s docking success.
Table 9. List of protein conformations and the small molecule cofactors that reside in or near each conformation’s binding site.

<table>
<thead>
<tr>
<th>Protein Conformation</th>
<th>Cofactors in Binding Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>4FU7</td>
<td>Sulfate</td>
</tr>
<tr>
<td>4FU8</td>
<td>Acetone, Succinate</td>
</tr>
<tr>
<td>4FU9</td>
<td>Succinate</td>
</tr>
<tr>
<td>4FUB</td>
<td>Succinate, Glycerol</td>
</tr>
<tr>
<td>4FUC</td>
<td>Succinate</td>
</tr>
<tr>
<td>4FUD</td>
<td>Sulfate</td>
</tr>
<tr>
<td>4FUE</td>
<td>Succinate</td>
</tr>
<tr>
<td>1OWE</td>
<td>none</td>
</tr>
<tr>
<td>1GJ7</td>
<td>none</td>
</tr>
</tbody>
</table>

Failure analysis: 2UP and 6UP

Ligands 2UP and 6UP had the worst scoring success of the ligand set and consistently failed in ensemble docking. This could have been because HADDOCK was scoring unacceptable clusters exceptionally well or was scoring acceptable clusters poorly. The small size of the ligands may have reduced the intermolecular interactions made between the ligand and the protein thereby allowing it to reach poses or binding locations that gave a better score but an unacceptable structure. Visually analyzing the failed docked structures show 2UP and 6UP bound in more variable poses and locations than other ligands in the set. The small ligands were docked with rotations around two main axes and could access more binding sites along the protein’s surface outside of the binding pocket. This supports the hypothesis that HADDOCK docked the small ligands into non-native binding sites with greater variability because of the reduced intermolecular interactions.

Failure Analysis: Ligand 132

As presented in the Results section, ligand 132 had the worst sampling success. HADDOCK often was not able to generate even one acceptable cluster when ligand 132 and a protein conformation were submitted. One possible reason for the poor sampling success may be the difference between the native protein conformation 1GJ7 and the other conformations. The graphs presented in Figure 10 show how different the binding sites of any two urokinase conformations are from each other. Though the data points are not labeled to indicate which conformation they represent, no point exceeds 0.3 Å site RMSD (ir-RMSD). This value is smaller than most of the ir-RMSD values between conformations of other proteins in the Astex diverse set. As such, urokinase has a binding site that is less variable than that of other proteins. It is notable that the data points in the graph for 1GJ7 are shifted to higher ir-RMSD values than any other graph, indicating that 1GJ7 has the most different binding site of the conformations in my set (Figure 10). Because urokinase has a particularly inflexible binding site, movement of the
binding site residues may be detrimental to docking success. 1GJ7 has a binding site that has changed to accommodate the bulky ligand 132, likely causing the higher ir-RMSD values. Figure 12 shows a per-residue ir-RMSD analysis of 1GJ7 and 4FU8. The crystal structure ligand and a failed docking into 4FU8 are shown in both panels for reference. The distance between the top and bottom highlighted residues appears to be narrower in 4FU8 than 1GJ7 and the residue on the right is pushed further right in 4FU8. The differences in the highlighted residues between the 1GJ7 and 4FU8 conformations may have forced the ligand toward the right side of the binding pocket when the ligand was docked into the non-native conformation 4FU8, ultimately resulting in a failed docking.

Figure 12. Residue-specific ir-RMSD between reference conformation 1GJ7 and mobile conformation 4FU8. The images on the right are a visual representation of the movement described in the ir-RMSD value. The top image shows the crystal structure ligand 132 (Orange) and a failed docking of ligand 132 (Blue) on the surface of the 1GJ7 conformation. The lower image shows the ligands on the surface of the mobile conformation 4FU8. The failed docking shown by the Blue ligand was performed with the 4FU8 conformation. The residues with an ir-RMSD of >0.250 are highlighted (Yellow) to show how their change between conformations may have affected docking.

In addition to binding site residue movement, the exclusion of some 1GJ7-132 contact residues from the AIRs residue set may have hurt sampling success. Ligand 132 had an exceptionally large 3.9 Å contact residue set, as denoted in Table 2. Several residues inside the binding pocket and on the protein surface to the left of the pocket that were in the 1GJ7-132 contact residue set but were excluded from the AIRs set (Figure 13, yellow). If these additional residues were included in the AIRs files, ligand 132 may have been able to dock with a higher
sampling success rate. Expanding the AIRs to 10 Å to include all of the residues in the binding site likely would have improved sampling success by allowing more of the crystal structure’s contact residues to fulfil the distance restraints.

Figure 13. 132-1GJ7 crystal structure with highlighted active residues. Ligand 132 is shown in its native conformation with the residues included in AIRs (Red) and the residues in the ligand 132 contact residue set that were excluded from the AIRs (Yellow).

Other failed runs show the double ring structure in ligand 132 docked correctly, nearly overlapping the crystal structure (Figure 14). However, the two large six-membered rings that point out of the protein are connected by rotatable bonds. Very small changes to the rotation of these bonds can determine which side of the septum this end of the ligand sits on and can make the i-RMSD unacceptable (3.140 Å). HADDOCK’s output clusters containing ligand 132 have a variety of angles around these bonds with some realistically simulating the crystal structure. Because the side of the ligand that binds to the residues in the pocket is almost correct, the i-RMSD increase caused by the other end may not be entirely representative of the docked ligand’s ability to inhibit urokinase’s function. These failed dockings may be false negatives that indicate the ligand does not bind well to the protein when it actually would in vivo.
Figure 14. Crystal structure ligand 132 (Orange) and a HADDOCK docked ligand 132 (Cyan) on the surface of 4FUE. The double ring structures of the ligands are nearly identical when they sit in the binding pocket. The main difference that caused the i-RMSD to be unacceptable for this structure (3.140 Å) is in the end that points out of the protein structure. This end consists of two large six-membered rings connected by rotatable bonds, allowing it to achieve orientations that would increase the i-RMSD value.

One final reason that ligand 132 failed may have been that 132 does not contain the core naphthyl-amidine functional group. Instead, it has a group with a nitrogen atom in a five-membered ring (N₃). In both the native and the docked structures, the N₁ and N₂ nitrogen groups form hydrogen bonds with ASP 192, SER 193, and GLY 221 just as the other ligands in the set do. In the native structure, the N₃ nitrogen does not form hydrogen bonds with the surrounding residues. This additional nitrogen is slightly positively charged (δ = 0.256) and may be forming interactions with the protein that other ligands do not because they have entirely non-polar carbon atoms in this area.

The native conformation, 1GJ7, was seemingly exempt from ligand 132’s poor docking success. Not only could ligand 132 be docked into its native conformation in an acceptable structure, HADDOCK also assigned them fantastic scores (the best being -73.150). In fact, the 1GJ7 conformation had success when docking every ligand into it.
Comparison to GOLD

To quantify how successful HADDOCK is at docking urokinase, I have compared its success to that of the GOLD docking program. According to the information presented in the Verdonk et al. article\textsuperscript{11}, the researchers were able to dock ligand 675 into five non-native urokinase protein conformations (1C5X, 1F5L, 1GJD, 1OWD, 1OWH) with a top 1 scoring success rate of 9\% using GOLD. Using HADDOCK, ligand 675 was successfully redocked into the native 1OWE structure and was assigned the best score. Ligand 675 was also docked into 5/8 non-native urokinase conformations and all five were top scoring. This indicates a 62.5\% top 1 scoring success rate, compared to GOLD’s 9\%. Additionally, none of the three failed non-native conformations had scores better than the best successful docking. Therefore, ensemble docking with HADDOCK was successful for ligand 675. Though this only includes dockings of urokinase and is not entirely indicative of either HADDOCK or GOLD, it is worth noting that HADDOCK ensemble docking performed 6.94 times better than GOLD did in a published article.

Parameters and Protocol

Introducing the Buried Site settings did not significantly improve sampling success, scoring success, or ensemble docking success. In most cases, Buried Site settings actually hurt success. The diminished intermolecular interactions in the Buried Site settings allowed the ligand to bind in more poses that were unacceptable. This creates more clusters overall and many of these additional clusters were unacceptable.

Docking urokinase may not require Buried Site settings because of the consistent shape of its binding site amongst different conformations. The measured ir-RMSD values demonstrate this consistency (Figure10). The distance between the backbone atoms in the binding sites of any two urokinase conformations was never more than 0.3 Å. This demonstrates that urokinase may have a particularly rigid binding site and introducing the Buried Site settings may model it as unrealistically accommodating to ligands thereby producing good scores for unacceptable structures.

Conclusions

Overall, the dockings run with Standard settings performed better than those run with Buried Site settings. Because Buried Site settings reduce intermolecular interactions to 1\% of the Standard settings and eliminated van der Waals forces, they allow the ligand to bind to the protein with less consideration for any repulsion around the binding site. Using Buried Site settings may be removing the intermolecular forces around the urokinase binding site that are crucial for inhibitors to bind with the correct pose.

The sampling success with New 1000 Standard settings was very high. It was near perfect with all but two ligands and the lowest case was still 4/9 (44\% success). Further, the sampling success column exactly matched the Top 3 scoring success column. HADDOCK in its
current form consistently docks urokinase inhibitors in acceptable structures and scores an acceptable cluster in the top 3 scores. This shows that HADDOCK, when combined with supplemental outside information, could be used to identify how a ligand binds to a protein. Finally, HADDOCK had 62.5% top 1 scoring success for ligand 675, comparable to GOLD’s 9% in the Verdonk et al. article.

Ensemble docking success for a ligand was determined by comparing the best scoring cluster produced from that ligand docked into each protein conformation. If the best scoring protein-ligand combination was also an acceptable structure, then the ensemble docking was successful. Standard settings produced successful ensemble docking for 6/8 ligands attempted with only ligands 2UP and 6UP failing the ensemble docking.

As was discussed in the Discussion section, ligand size and shape may have contributed to diminished sampling success and scoring success. HADDOCK has trouble docking the largest ligand, 132, into non-native conformations. This may be attributed to several factors including the difference in position of binding site residues, the lack of naphthyl-amidine functional group, and contact residues excluded from the AIRs set. HADDOCK also has trouble scoring the smallest ligands, 2UP and 6UP, correctly which is likely attributed to diminished intermolecular penalties due to their small size.

HADDOCK’s scoring function currently cannot generate comparable scores across different ligands. Ideally, ligands that have better affinity for urokinase will also have a better HADDOCK score and you could use the HADDOCK score to determine which ligand would be the best inhibitor to urokinase. From these data, there is no strong correlation between HADDOCK scores and affinity values. This may be a result of the relatively small range of affinity values in this set of urokinase inhibitors (0.013 μM of 132 to 5.91 μM of 2UP). When non-binding ligands (affinities greater than 100 μM) were docked into urokinase they typically had worse HADDOCK scores when using Standard settings. HADDOCK’s scoring function needs to be improved so it can more easily identify non-binding ligands and can differentiate urokinase inhibitors according to their affinity. HADDOCK is not alone in these scoring shortcomings. Creating an accurate scoring function is a long-standing challenge across the docking field and will continue to be pursued by researchers.

**Future Research**

Several extensions to this project might be pursued to further explore HADDOCK’s applicability to urokinase. First, increasing the number of residues included in the AIRs might affect HADDOCK’s accuracy. In this project, AIRs were designed to include the residues within 3.9 Å of the ligand 239 in its crystal structure. This relatively small set of residues may not have been broad enough to dock the largest ligand, 132, accurately. An alternative method used in the Grinstead lab to create AIRs is to include all residues within 10 Å of the docked ligand. This provides HADDOCK with a more expansive binding site than the 3.9 Å distance. Increasing the
residues in the AIRs could affect HADDOCK’s docking success with urokinase ligands by providing more residues to satisfy the distance restraints.

Urokinase was more successful when using the Standard settings in my project. It could be beneficial to continue docking urokinase inhibitors with a wider range of affinities with the Standard settings. Using a wider range of affinities may give insight into how to improve the scoring function of HADDOCK across various ligands. Additionally, it is worth noting that all of the inhibitor ligands in this set were naphthyl-amidine derivatives. Ligands with a different core functional group could have different success rates when docking with HADDOCK.

Finally, 1GJ7’s consistently good scoring and sampling success may be attributed to the difference that its binding site has from other protein conformations. Particularly, the difference in location of the ASP 50, HIS 94, GLN 195, and SER 198 residues may explain this success. The Astex diverse set\(^6\) contains several protein conformations with a greater ir-RMSD vs 1OWE than the conformations in my set. Considering the 1C5X (ir-RMSD = 0.746) and 1GJD (ir-RMSD = 0.697) urokinase conformations would be beneficial to work with greater binding site mobility than was presented in my set of protein conformations. Any future dockings involving urokinase should consider how site conformation may influence docking success.

References


(16) Mahmood, N.; Mihalciou, C.; Rabbani, S. A. Multi-Faceted Role of the Urokinase Plasminogen Activator (UPA) and Its Receptor (UPAR); Diagnostic, Prognostic, and Therapeutic Applications. Front. Oncol. 2018, 8, 24.


Supplemental Information

Figure 15. Results from HADDOCK dockings of 1UP into urokinase protein conformations.

<table>
<thead>
<tr>
<th>Docked ligand: 1UP</th>
<th>Native Protein Conformation: 4FU7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Docked ligand: 1UP Native ligand: 1UP ir-RMSD vs 4FU7: 0.000</td>
<td></td>
</tr>
<tr>
<td>Docked ligand: 2UP Native ligand: 2UP ir-RMSD vs 4FU7: 0.155</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>New Protein Conformation</th>
<th>HADDOCK score vs i-RMSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>4FU7</td>
<td><img src="graph.png" alt="Graph" /></td>
</tr>
<tr>
<td>4FU8</td>
<td><img src="graph.png" alt="Graph" /></td>
</tr>
</tbody>
</table>
4FU9

Native ligand: 675
ir-RMSD vs 4FU7: 0.119

4FUB

Native ligand: 4UP
ir-RMSD vs 4FU7: 0.120

4FUC

Native ligand: 239
ir-RMSD vs 4FU7: 0.143
4FUD

Native ligand: 6UP
ir-RMSD vs 4FU7: 0.094

4FUE

Native ligand: 7UP
ir-RMSD vs 4FU7: 0.142

1OWE

Native ligand: 675
ir-RMSD vs 4FU7: 0.156
Figure 16. Results from HADDOCK dockings of 2UP into urokinase protein conformations.

Docked ligand: 2UP
Native Protein Conformation: 4FU8

New Protein Conformation

<table>
<thead>
<tr>
<th>HADDOCK score vs i-RMSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
</tr>
</tbody>
</table>

| New 1000 Standard | New 1000 Buried Site |

Native ligand: 1UP
ir-RMSD vs 4FU8: 0.155

Native ligand: 132
ir-RMSD vs 4FU7: 0.224
**4FU8**

Native ligand: 2UP
ir-RMSD vs 4FU8: 0.000

**4FU9**

Native ligand: 675
ir-RMSD vs 4FU8: 0.170

**4FUB**

Native ligand: 4UP
ir-RMSD vs 4FU8: 0.156
4FUC

Native ligand: 239
ir-RMSD vs 4FU8: 0.148

4FUD

Native ligand: 6UP
ir-RMSD vs 4FU8: 0.121

4FUE

Native ligand: 7UP
ir-RMSD vs 4FU8: 0.167
**1OWE**

Native ligand: 675
ir-RMSD vs 4FU8: 0.189

**1GJ7**

Native ligand: 132
ir-RMSD vs 4FU8: 0.248
Figure 17. Results from HADDOCK dockings of 675 into urokinase protein conformations.

Docked ligand: 675
Native Protein Conformation: 4FU9 and 1OWE

<table>
<thead>
<tr>
<th>New Protein Conformation</th>
<th>HADDOCK score vs i-RMSD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>4FU7</strong></td>
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</tr>
<tr>
<td>Native ligand: 1UP</td>
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</tr>
<tr>
<td>ir-RMSD vs 4FU9: 0.119</td>
<td></td>
</tr>
<tr>
<td>ir-RMSD vs 1OWE: 0.156</td>
<td></td>
</tr>
</tbody>
</table>

| **4FU8**                 |                         |
| Native ligand: 2UP       |                         |
| ir-RMSD vs 4FU9: 0.170   |                         |
| ir-RMSD vs 1OWE: 0.189   |                         |
**4FU9**

Native ligand: 675  
ir-RMSD vs 4FU9: 0.000  
ir-RMSD vs 1OWE: 0.154

**4FUB**

Native ligand: 4UP  
ir-RMSD vs 4FU9: 0.121  
ir-RMSD vs 1OWE: 0.143

**4FUC**

Native ligand: 239  
ir-RMSD vs 4FU9: 0.107  
ir-RMSD vs 1OWE: 0.150
**4FUD**

Native ligand: 6UP  
ir-RMSD vs 4FU9: 0.139  
ir-RMSD vs 1OWE: 0.168

**4FUE**

Native ligand: 7UP  
ir-RMSD vs 4FU9: 0.140  
ir-RMSD vs 1OWE: 0.146

**1OWE**

Native ligand: 675  
ir-RMSD vs 4FU9: 0.154  
ir-RMSD vs 1OWE: 0.000
Figure 18. Results from HADDOCK dockings of 4UP into urokinase protein conformations.

Docked ligand: 4UP
Native Protein Conformation: 4FUB

<table>
<thead>
<tr>
<th>New Protein Conformation</th>
<th>HADDOCK score vs i-RMSD</th>
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<tbody>
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<td>1GJ7</td>
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<td></td>
</tr>
<tr>
<td>Native ligand: 132</td>
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<td>ir-RMSD vs 4FU: 0.203</td>
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<tr>
<td>ir-RMSD vs 1OWE: 0.238</td>
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<td>4FU7</td>
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<td></td>
</tr>
<tr>
<td>Native ligand: 1UP</td>
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</tr>
<tr>
<td>ir-RMSD vs 4FUB: 0.120</td>
<td></td>
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</tbody>
</table>

New 1000 Standard
New 1000 Buried Site
**4FU8**

Native ligand: 2UP
ir-RMSD vs 4FUB: 0.156

**4FU9**

Native ligand: 675
ir-RMSD vs 4FUB: 0.121

**4FUB**

Native ligand: 4UP
ir-RMSD vs 4FUB: 0.000
**4FUC**

Native ligand: 239
ir-RMSD vs 4FUB: 0.147

**4FUD**

Native ligand: 6UP
ir-RMSD vs 4FUB: 0.104

**4FUE**

Native ligand: 7UP
ir-RMSD vs 4FUB: 0.137
Native ligand: 675
ir-RMSD vs 4FUB: 0.143

Native ligand: 132
ir-RMSD vs 4FUB: 0.224
**Figure 19.** Results from HADDOCK dockings of 239 into urokinase protein conformations.

<table>
<thead>
<tr>
<th>Docked ligand: 239</th>
<th>Native Protein Conformation: 4FUC</th>
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<tbody>
<tr>
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<td>Native ligand: 1UP</td>
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<td>ir-RMSD vs 4FUC: 0.143</td>
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<td>Native ligand: 2UP</td>
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<tr>
<td>ir-RMSD vs 4FUC: 0.148</td>
<td><img src="image" alt="Graph" /></td>
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</table>
4FU9
Native ligand: 675
ir-RMSD vs 4FUC: 0.107

4FUB
Native ligand: 4UP
ir-RMSD vs 4FUC: 0.147

4FUC
Native ligand: 239
ir-RMSD vs 4FUC: 0.000
4FUD
Native ligand: 6UP
ir-RMSD vs 4FUC: 0.144

4FUE
Native ligand: 7UP
ir-RMSD vs 4FUC: 0.103

1OWE
Native ligand: 675
ir-RMSD vs 4FUC: 0.150
Figure 20. Results from HADDOCK dockings of 6UP into urokinase protein conformations.

**1GJ7**

Native ligand: 132
ir-RMSD vs 4FUC: 0.229

**4FU7**

Native ligand: 1UP
ir-RMSD vs 4FUD: 0.094
4FU8

Native ligand: 2UP
ir-RMSD vs 4FUD: 0.121

4FU9

Native ligand: 675
ir-RMSD vs 4FUD: 0.139

4FUB

Native ligand: 4UP
ir-RMSD vs 4FUD: 0.104
Native ligand: 239
ir-RMSD vs 4FUD: 0.144

Native ligand: 6UP
ir-RMSD vs 4FUD: 0.000

Native ligand: 7UP
ir-RMSD vs 4FUD: 0.132
**1OWE**

Native ligand: 675
ir-RMSD vs 4FUD: 0.168

**1GJ7**

Native ligand: 132
ir-RMSD vs 4FUD: 0.233
**Figure 21.** Results from HADDOCK dockings of 7UP into urokinase protein conformations.

Docked ligand: 7UP
Native Protein Conformation: 4FUE

<table>
<thead>
<tr>
<th>New Protein Conformation</th>
<th>HADDOCK score vs i-RMSD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>4FU7</strong></td>
<td></td>
</tr>
<tr>
<td>Native ligand: 1UP</td>
<td></td>
</tr>
<tr>
<td>ir-RMSD vs 4FUE: 0.142</td>
<td></td>
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<tr>
<td></td>
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<tr>
<td><strong>4FU8</strong></td>
<td></td>
</tr>
<tr>
<td>Native ligand: 2UP</td>
<td></td>
</tr>
<tr>
<td>ir-RMSD vs 4FUE: 0.167</td>
<td></td>
</tr>
</tbody>
</table>
4FU9
Native ligand: 675
ir-RMSD vs 4FUE: 0.140

4FUB
Native ligand: 4UP
ir-RMSD vs 4FUE: 0.137

4FUC
Native ligand: 239
ir-RMSD vs 4FUE: 0.103
4FUD
Native ligand: 6UP
ir-RMSD vs 4FUE: 0.132

4FUE
Native ligand: 7UP
ir-RMSD vs 4FUE: 0.000

1OWE
Native ligand: 675
ir-RMSD vs 4FUE: 0.146
Figure 22. Results from HADDOCK dockings of 132 into urokinase protein conformations.
4FU8
Native ligand: 2UP
ir-RMSD vs 1GJ7: 0.248

4FU9
Native ligand: 675
ir-RMSD vs 1GJ7: 0.203

4FUB
Native ligand: 4UP
ir-RMSD vs 1GJ7: 0.224
4FUC
Native ligand: 239
ir-RMSD vs 1GJ7: 0.229

4FUD
Native ligand: 6UP
ir-RMSD vs 1GJ7: 0.233

4FUE
Native ligand: 7UP
ir-RMSD vs 1GJ7: 0.233
Native ligand: 675
ir-RMSD vs 1GJ7: 0.238

Native ligand: 132
ir-RMSD vs 1GJ7: 0.000