Bversity
1st Ever Virtual College for Biotech

Name: Rajashree. U
Contact:rajashree5820@gmail.com
Mobile:6383331696
Title: Protein Structural analysis

Answer the following questions below and upload them in the google form.

1. You are targeting the P1 and P40/P90 proteins of Mycoplasma pneumoniae for inhibiting its pathogenic activity and comparative analysis.

    a. Mention potential binding sites on P1 and P40/P90 that could be targeted for inhibition.

    b. Discuss the potential challenges and limitations of targeting P1 and P40/P90 proteins for Mycoplasma pneumoniae treatment.

    c. Discuss the significance of this research in developing novel therapeutic strategies for Mycoplasma pneumoniae infections.

PROTEIN STRUCTURE ANALYSIS

The aim is to find potential binding sites on P1 and P40/P90 that could be targeted for inhibition. The P1 and P40/P90 proteins are mycoplasma pneumonia proteins. *Mycoplasma pneumoniae* is the most common pathogen causing respiratory tract infection, and the P1 protein on its adhesion organelle plays a crucial role during the pathogenic process. Currently, there are many studies on P1 and receptors on host cells, but the adhesion mechanism of P1 protein is still unclear. Both p1 and p40/p90 protein are cell adhesion protein

P1 Adhesion Protein of Mycoplasma pneumoniae:

The P1 adhesion protein is a crucial virulence factor of Mycoplasma pneumoniae, a tiny bacterium lacking a cell wall that causes primary atypical pneumonia. This protein plays a vital role in the bacterium's ability to adhere to and invade human respiratory epithelial cells, initiating the infection process.

P1 is a large, 170 kDa protein with a complex structure. It consists of several domains with distinct functions.

N-terminal head domain This domain interacts with various host cell receptors, such as sialic acid and vimentin, mediating adhesion**.**

Central stalk domain: This elongated, flexible domain acts like a bridge, connecting the head domain to the transmembrane anchor.

The C-terminal transmembrane domain anchors P1 in the bacterial membrane, allowing it to form a stable attachment with the host cell surface. P1 plays a critical role in several steps of Mycoplasma pneumoniae infection.

P1 interacts with host cell receptors, enabling the bacterium to attach to the respiratory epithelium.

P1, along with other proteins, forms a gliding apparatus that allows the bacterium to move on the cell surface, facilitating further invasion.

P1 can mask other bacterial surface antigens, making it difficult for the immune system to recognize and attack the pathogen. So, Understanding P1's function is crucial for developing new strategies to combat Mycoplasma pneumoniae infections. Researchers are investigating:

P1-based vaccines targeting P1 could potentially prevent bacterial adhesion and infection.

Antimicrobial drugs inhibiting P1 function could be a novel approach to combatting antibiotic-resistant strains.

Diagnostic Detecting P1 in clinical samples could help diagnose Mycoplasma pneumoniae infections more accurately.

P1 adhesion protein is a fascinating example of how a single molecule can play a multifaceted role in bacterial pathogenesis. Understanding its intricate mechanisms paves the way for developing new interventions to combat this common and potentially serious respiratory infection.

P40/P90 Adhesion Protein: Mycoplasma pneumonia:

Alongside P1, another critical player in Mycoplasma pneumonia's infectious arsenal is the P40/P90 adhesion protein. This dynamic duo works in tandem to ensure the bacterium's successful invasion of human respiratory cells.

P40 and P90 are two separate proteins that often appear linked together, hence the combined P40/P90 designation.P40 is a smaller protein (40 kDa) with a single transmembrane domain anchoring it to the bacterial membrane.P90 is a larger protein (90 kDa) with several domains:

The N-terminal head domain binds sialic acid on host cell surfaces, mediating initial attachment.

The central domain links P40 and P90 and potentially interacts with other proteins.

The C-terminal domain interacts with P1, forming a stable adhesion complex.

P40/P90 adhesion protein of Mycoplasma pneumoniae structure. Together, P40/P90 and P1 form a transmembrane adhesion complex that strengthens the bacterium's grip on host cells. Adherence to P90's sialic acid binding initiates initial attachment, while P40/P90 interaction with P1 stabilizes the bond. Motility Like P1, P40/P90 contributes to the bacterium's gliding movement on the host cell surface.

Immune evasion P40/P90 may help mask other bacterial antigens, hindering immune recognition. Disrupting this complex could prevent M. pneumoniae adhesion and invasion. P40/P90-based vaccines Similar to P1, P40/P90 could be potential vaccine targets. Detecting P40/P90 in samples could improve diagnostic accuracy.P40/P90 adhesion protein demonstrates the intricate teamwork employed by M. pneumoniae for successful infection. Studying its role opens exciting avenues for developing novel interventions to combat this prevalent pathogen.

Ligands for Adhesion Domains:

P1 N-terminal head domain:

Antibodies targeting sialic acid-binding pocket could block host cell adherence.

Small molecules mimicking host cell receptors like vimentin could competitively inhibit binding.

P90 N-terminal head domain:

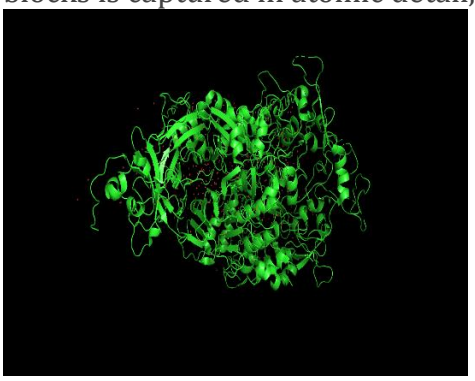
Sialic acid analogs could compete for binding and prevent adhesion. Peptides or peptidomimetics mimicking the host cell receptor binding site could act as inhibitors.

The PDB structures of 6RC9 (P1 protein) and 6RJ1 (P40/P90) are taken from PDB databank here for modelling .

6RC9 (P1 protein) :

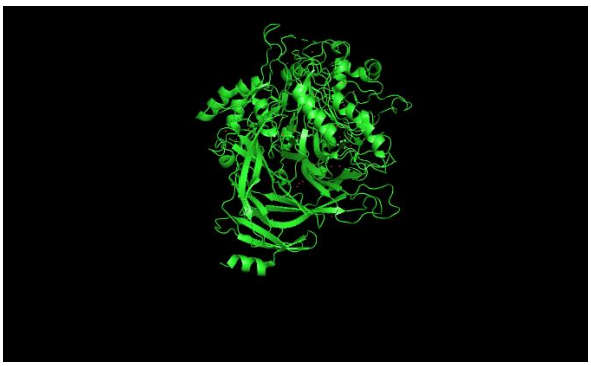
6RC9 offers a fascinating glimpse into the molecular architecture of P1, a key adhesion protein employed by the respiratory pathogen Mycoplasma pneumoniae to latch onto human cells. This detailed structural information, obtained through X-ray crystallography. Derived from Mycoplasma pneumoniae

M129 and expressed in Escherichia coli BL21(DE3), this protein structure reveals a remarkable assembly of 1,339 amino acid residues, weighing in at a hefty 159.79 kDa. The intricate arrangement of these building blocks is captured in atomic detail, encompassing 10,929 atoms within a single, continuous protein chain.



6RJ1 (P40/P90)

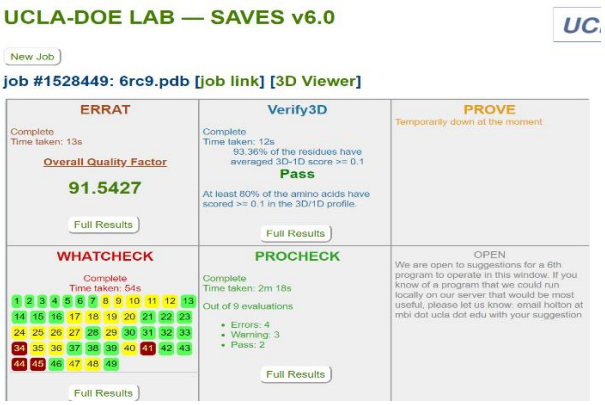
6RJ1of Mycoplasma pneumonia's infectious strategy: the N-domain of the P40/P90 adhesion protein. This detailed structural information, obtained through X-RAY DIFFRACTION Derived from Mycoplasma pneumoniae M129 and expressed in Escherichia coli BL21(DE3)The protein structure composed of 1,618 amino acid residues, assembled into a complex tapestry of 12,623 atoms. This translates to a total weight of 208.65 kDa. The N-domain is found as a single protein chain, designated as chain A and chain B. The amino acid sequence of this N-domain stretches for 976 residues, each playing a specific role in shaping its structure and function.

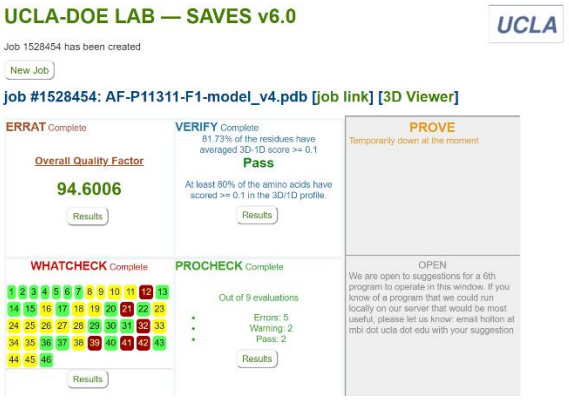


Alpha fold

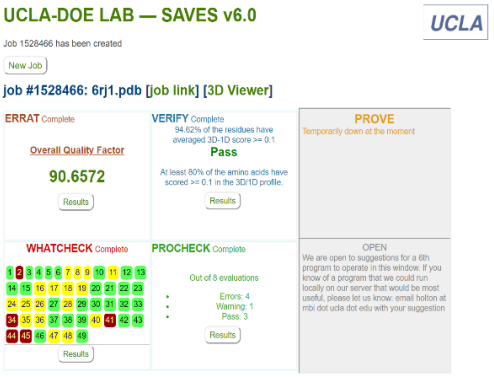
AlphaFold is a powerful new tool that is revolutionizing the field of protein structure prediction. It can be used to model the structures of proteins with high accuracy, even for those that are difficult to crystallize or resolve using traditional methods. This has several advantages for research. Alpha Fold often produces models that are more accurate than experimental structures, especially for challenging proteins. This means that we can now get a better understanding of how these proteins work. It can be used to model the structures of proteins that are not yet represented in the PDB.
AlphaFold can generate multiple models for a single protein, which can represent different conformational states or potential binding partners. This allows us to study how proteins move and interact with other molecules.

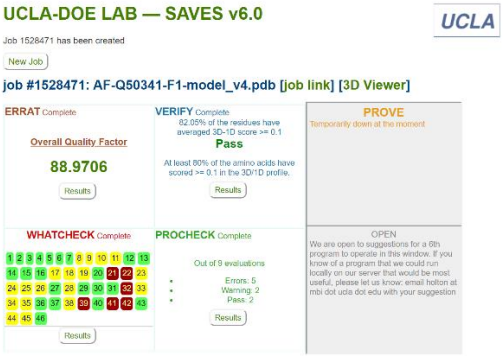
for then the structure obtained is checked for the overall quality score from saves. To compare models before and after Alpha fold the one with highest quality score is taken for further modelling. The quality score of Alphafold model was 94.6006 and from PDB was 91.5427 so, the Alphafold model was taken for 6RC9. Whereas the quality score of the Alphafold model was 88.9706 and from pdb was 90.6572 so, here pdb model was taken for further process.





The above are images from saves server representing quality factor for structures from PDB and Alphafold for 6rc9





The above are images from saves server representing quality factor for structures from PDB and Alphafold for 6RJ1

phyre2

phyre2 was done to get the homology models. the highly identical one is taken for further analysis. Phyre2 is a powerful protein structure prediction tool that uses advanced algorithms and multiple sequence alignments to create accurate models. 100% identity indicates a perfect match between the model's amino acid sequence and the known protein sequence, suggesting a high degree of structural similarity. Phyre2 can potentially model proteins that lack experimental structures in the PDB, expanding the scope of proteins for which docking can be performed. Phyre2 can generate multiple models for a single protein, representing different conformations or binding states, potentially capturing a broader range of potential interactions. While Phyre2 models can be highly accurate, especially with 100% identity, they

may still contain minor errors or uncertainties compared to experimental structures. Careful validation of docking results is crucial. With the homology model obtained from Phyre 2 further Docking is done with PyRx.

The structure is pre-processed with PyMOL software:
The steps involved are as follows:

- Firstly the water molecules in the structure and if any bounded atoms are present they are removed.
- Then the file is saved in zero state with no charges for docking.

PyRx

PyRx is a flexible, open-source software package designed for protein-ligand docking and virtual screening. It offers a user-friendly interface. It Prepare proteins and ligands Optimize structures, identify binding pockets, and generate flexible conformations. Perform docking simulations, Employ various algorithms to explore potential binding modes and assess their scoring. Visualize docked complexes, calculate binding affinities, and identify key interactions. Optimizing lead compounds by Refining ligand structures for improved binding and potentially unlock new drug candidates' empowers researchers to delve deeper into the intricate world of molecular interactions, unlocking the secrets of protein function and paving the way for the development of innovative drugs and therapies. So, when the curtains rise on the next chapter of drug discovery.

Docking with PyRx:

- This involves loading protein, then making it macromolecule through adding autodock which brings back its water molecules and charges converting it into PDBQT format.
- Edit preference to workspace location where we want our file to be saved automatically after docking.
- Then the ligand is loaded through Open babel in SDF format.It is minimized and and converted to PDBQT format.
- The next step is to dock the protein-ligand with Vina Wizard by selecting the ligand and macromolecule that is needed to be docked.
- The process is forwarded to set grid box to the region where we need our docking to take place and the docking will start.
- After completion of the process, we can the image of the ligand component being docked to the macromolecule and the results give values of rmsd (Root Mean Square Deviation) and Binding affinity(Kcal/Mol)
- With which we can infer the interactions of protein and ligand.
- We can see the results saved in a location which we set at first.
- Now we can open the macromolecule and ligand in pymol and visualize how they are interacting.
- The ligand is selected and renamed with ligand_name. we can also give contrasting colours to ligand and macromolecule for better visualization
- We are able to get annotations of Interaction such as :

- The residues that are at 5 Å distance are shown by using the command in the pymol console : Command: Show sticks, byres all within 5 of ligand_name.
- The residues that are making polar contacts with ligand.
- The preferred residues are selected and renamed as active-site, By labelling this we can see names of the residues in the active site and that making polar contacts.

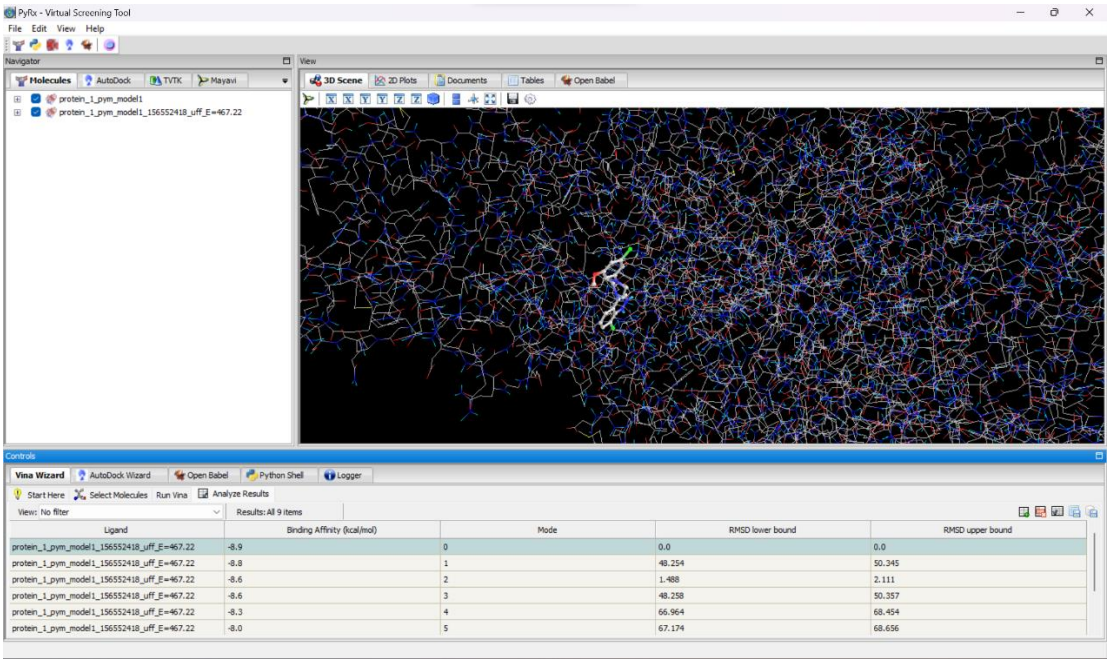
- By changing the colour of the active site we can see that the particular amino acids in sequence also highlighted.
- Finally, save this as a protein-ligand complex with its current state.

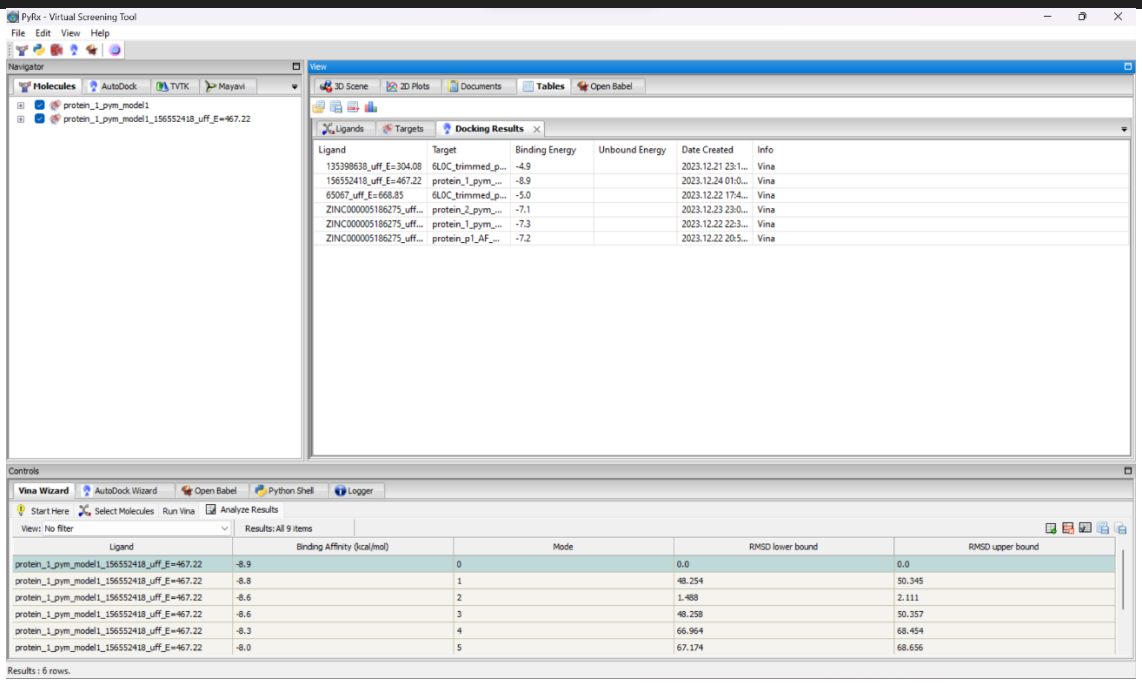
Discovery Studio Visualizer:

- It makes the process of visualizing protein-ligand interaction much easier.
- Just we can open the protein-ligand complex in the Discovery studio visualizer and see the interaction between ligand and residues by selecting Display receptor-ligand interaction
- It also gives the 2-D structure of residues interacting with ligands and the detail of bonds through which they are interacting.

Potential Binding sites :

P1 Protein PyRx Docking and inference:





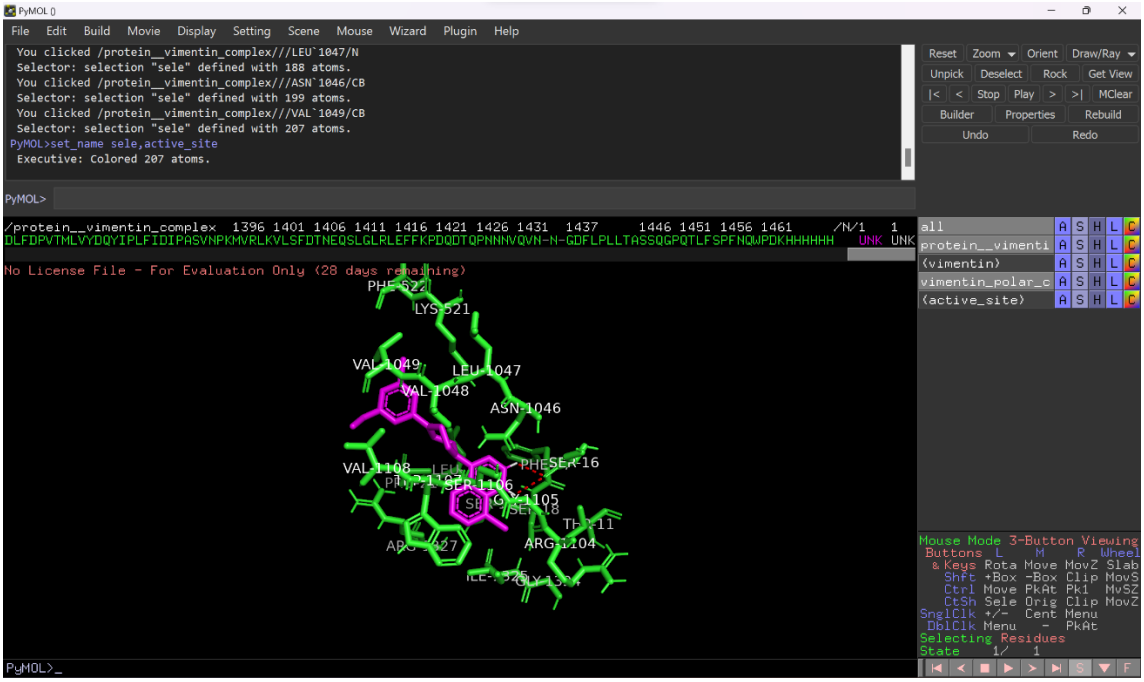
The docking was done between the P1 protein and Vimentin.

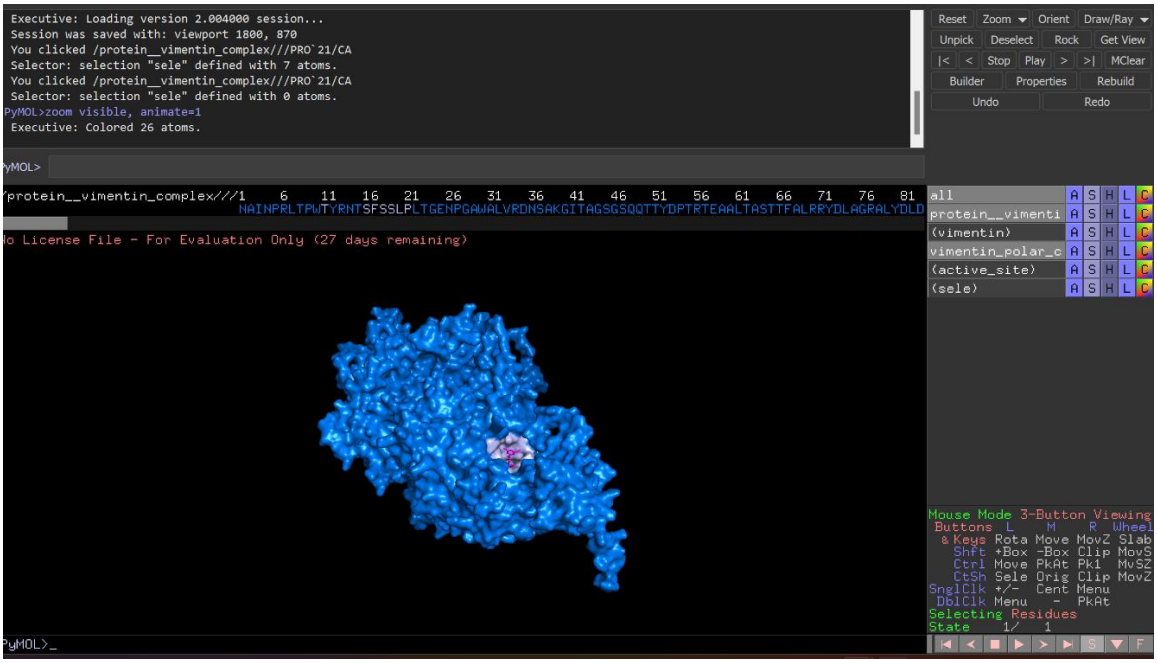
Binding affinity of -8.6:

This is a high binding affinity, suggesting a strong and favourable interaction between the protein and ligand. A value below -7 generally indicates good binding potential. This suggests that the ligand forms strong bonds with the protein, potentially leading to high potency and efficacy.

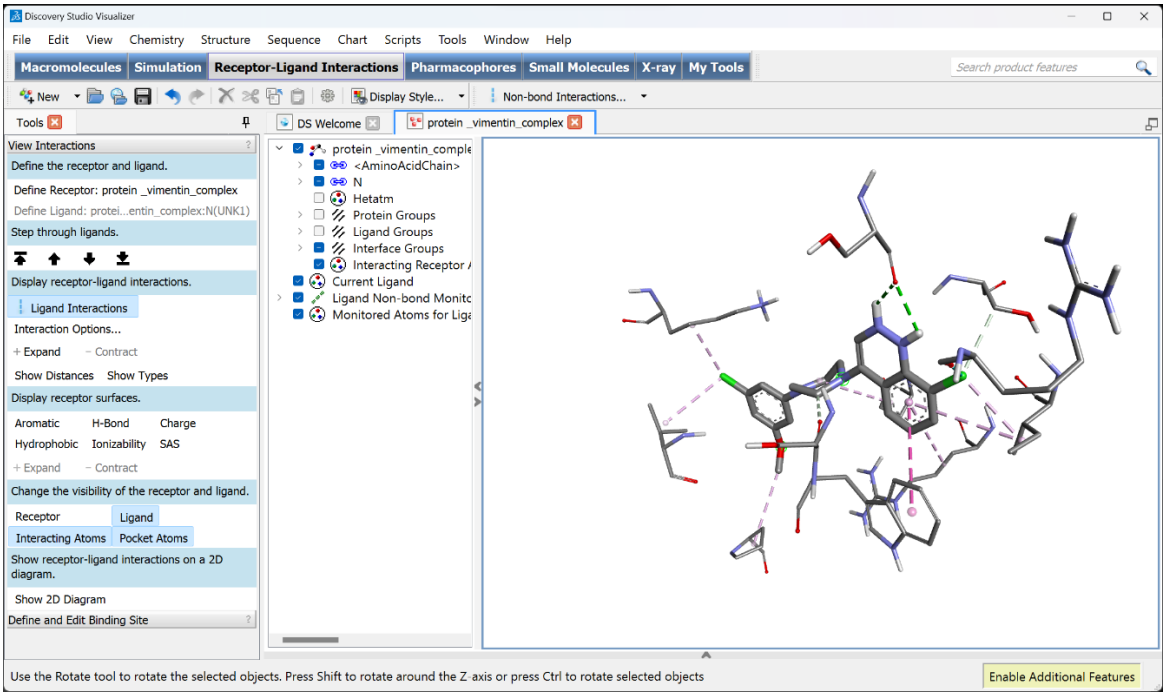
RMSD value of 1.488:

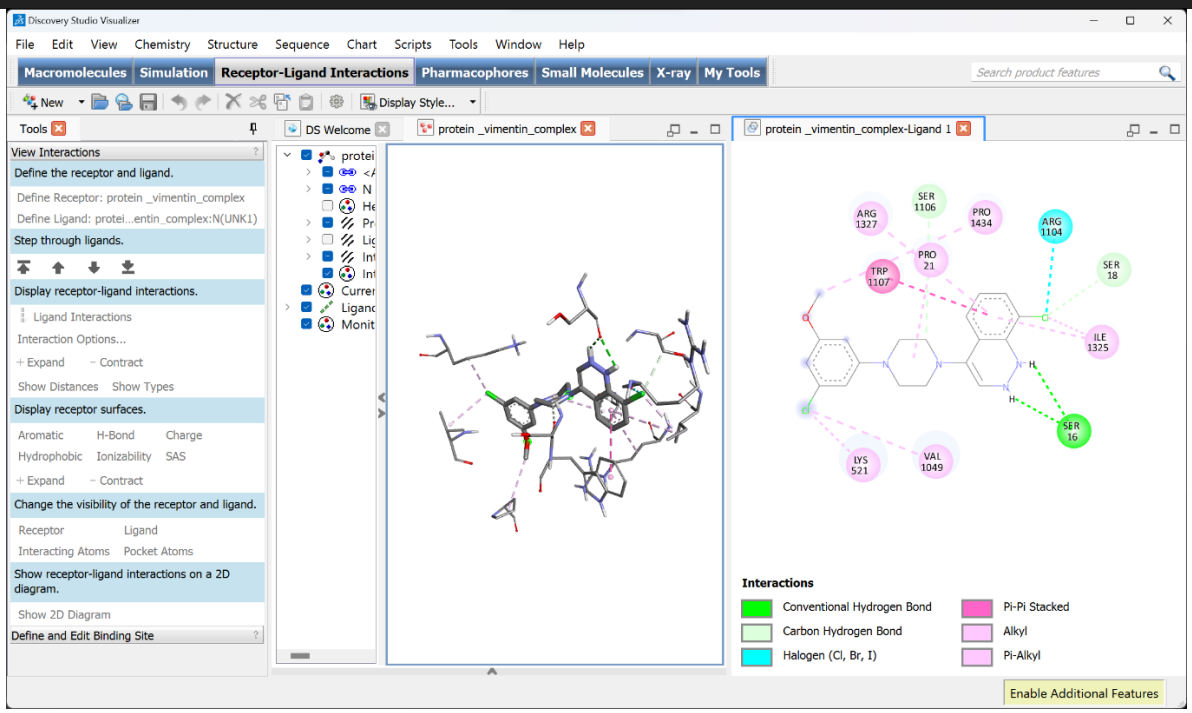
This is a low RMSD value, indicating a highly accurate and reliable docking pose. Ideally, RMSD values below 2 Å suggest a good fit within the binding pocket. This suggests that the predicted binding mode of the ligand closely aligns with its expected position within the protein, further supporting the strong binding affinity.





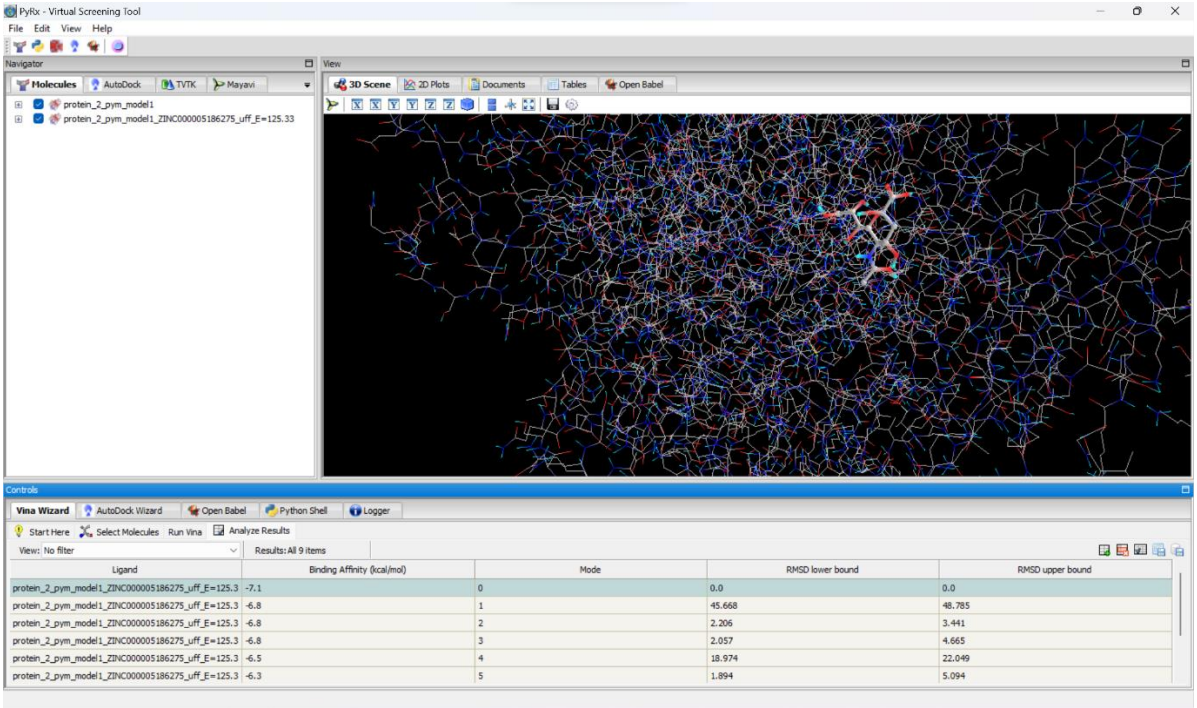
The above are pymol images showing the possible residues of potential binding sites for p1 protein and vimentin.

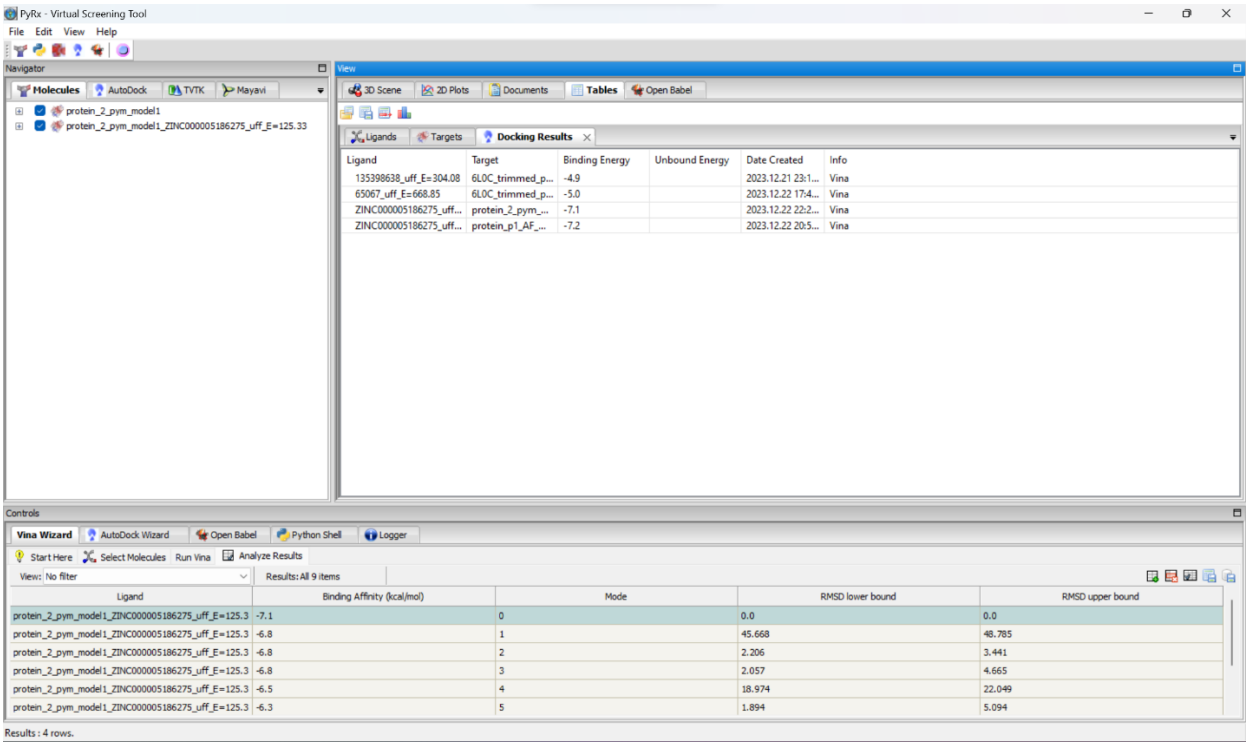




The above are 3D and 2D images from the Discovery studio visualizer give the interaction of residues with ligand vimentin.

Protein P40/P90 pyRx docking and inference:





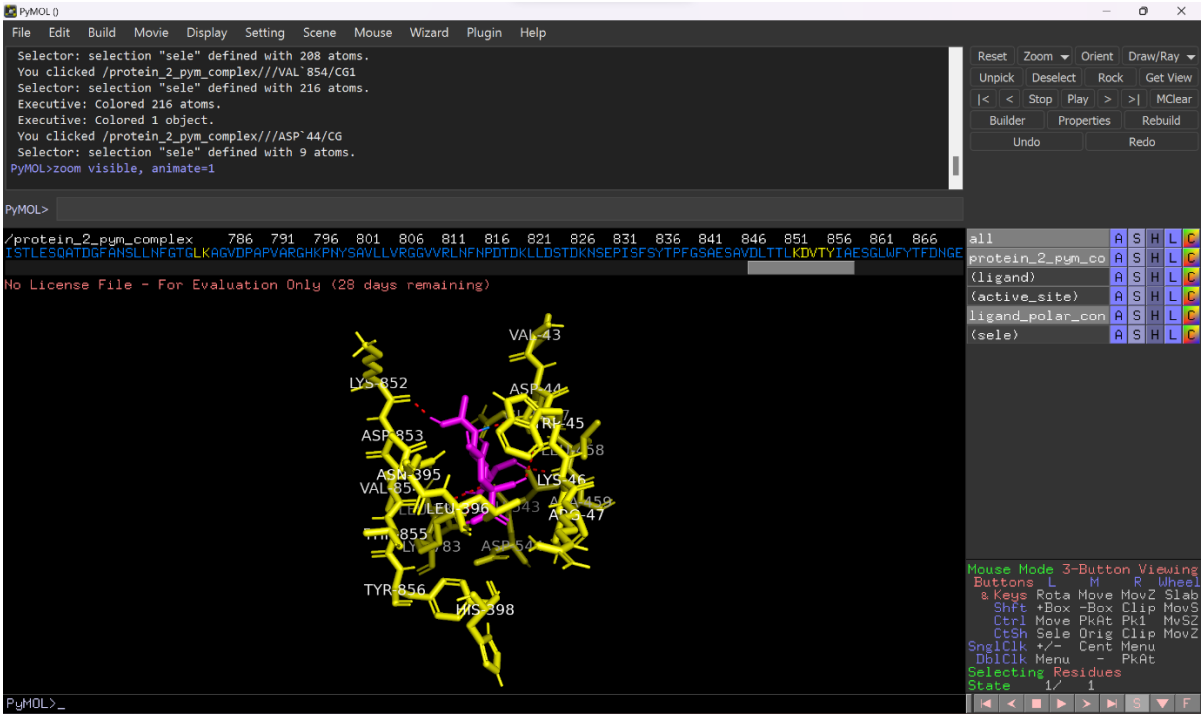
The above are images of protein-ligand interaction and the results obtained by docking.

Binding Affinity of -6.8:

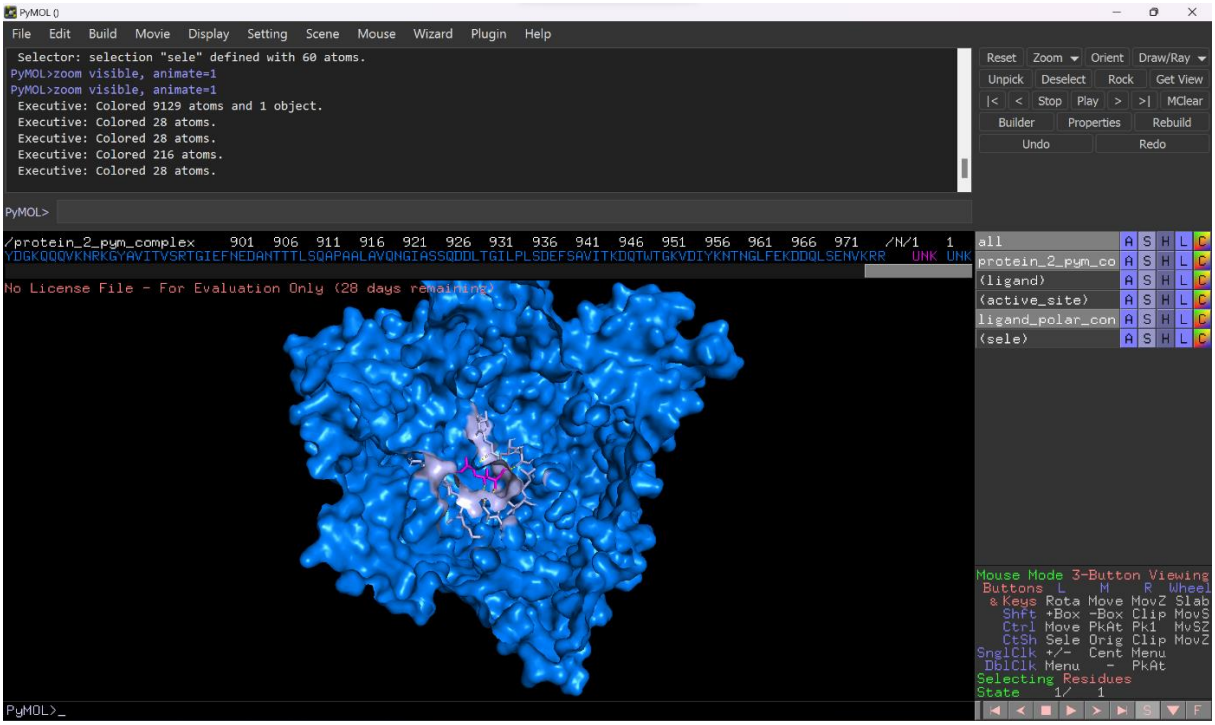
This value indicates a favorable binding interaction between the protein and ligand. While not the highest score achievable, it suggests a potentially significant binding strength.

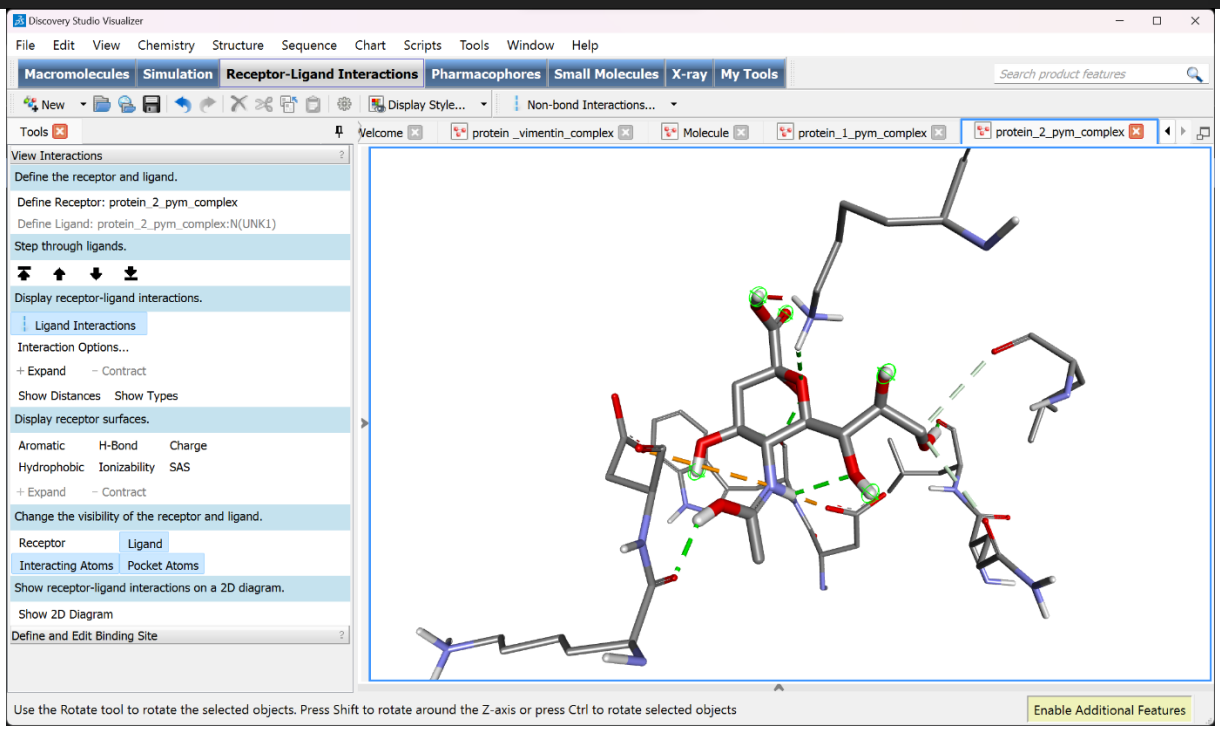
RMSD of 2.206:

This value falls within the typical range for a good docking pose (<2 Å), suggesting the ligand closely aligns with the predicted binding pocket.



The pymol images show the possible residues of potential binding sites for P40/P90 protein and Sialic acid




Discovery Studio Visualizer
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View Interactions
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Show receptor-ligand interactions on a 2D diagram.
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Define and Edit Binding Site
Use the Rotate tool to rotate the selected objects. Press Shift to rotate around the Z-axis or press Ctrl to rotate selected objects
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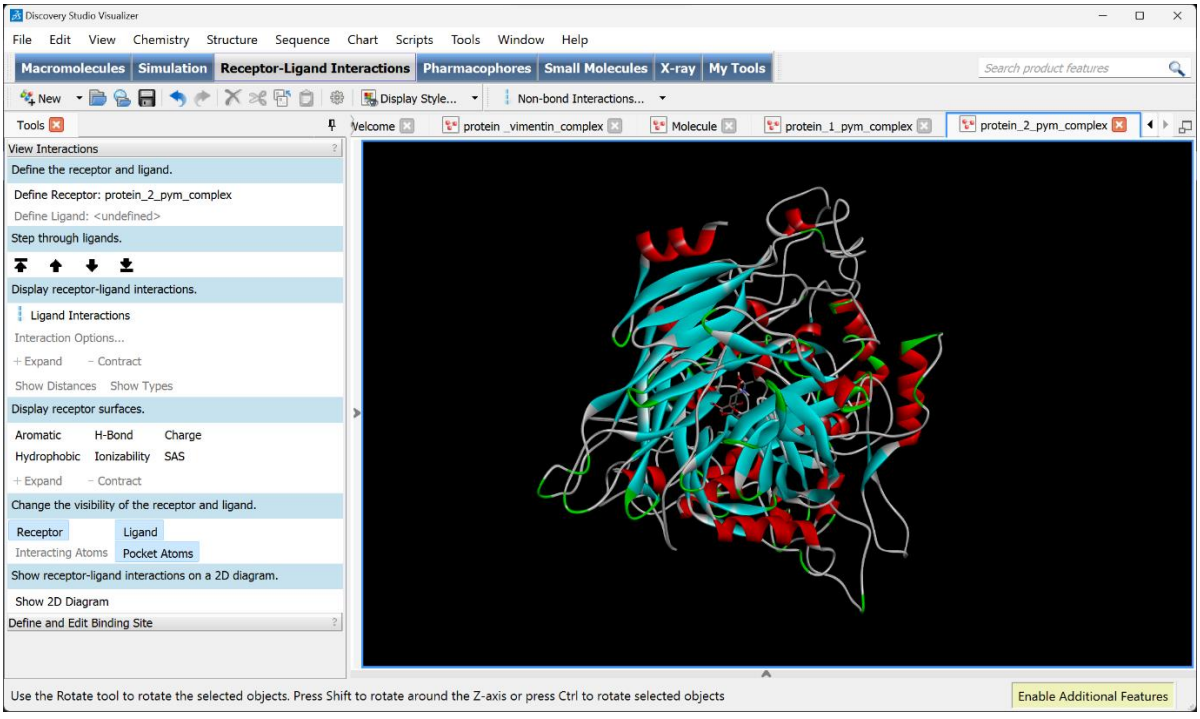
Discovery Studio Visualizer
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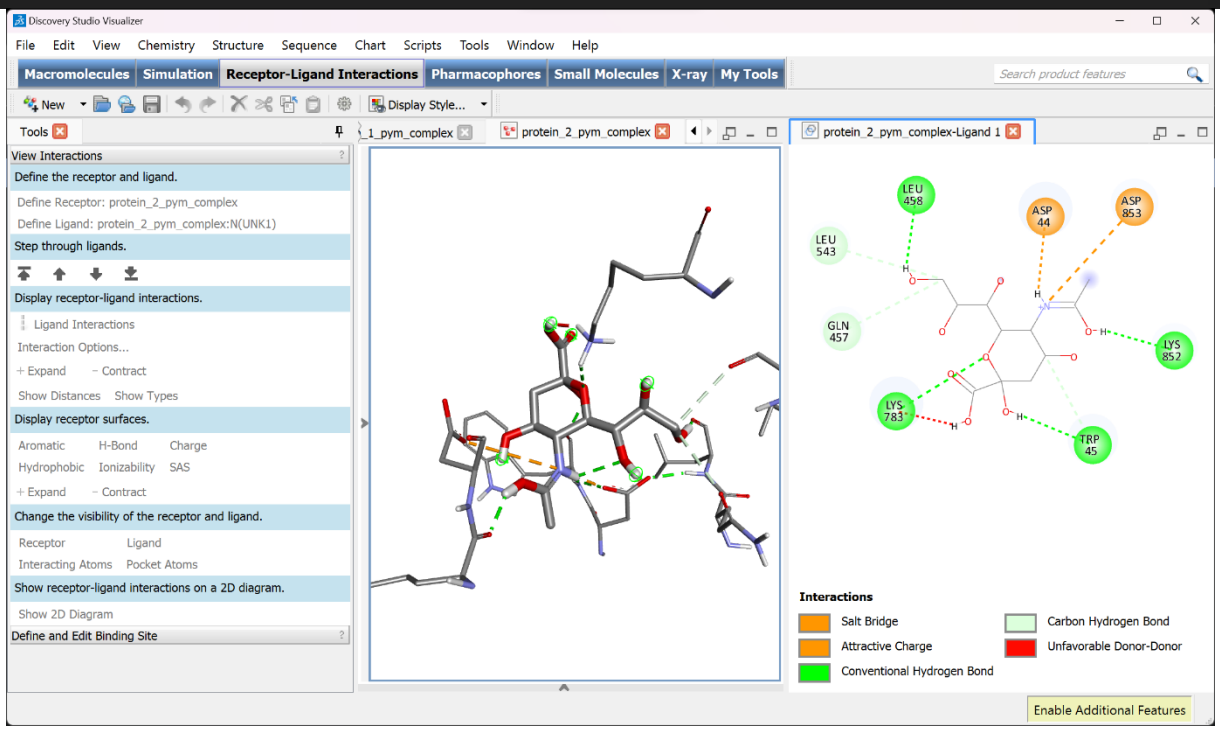

The above are images from the Discovery studio visualizer give 3D and 2D images of of residues in protein-ligand interaction and the details of bonds through which they are interacting

From the above results, the residues obtained by docking can be considered as one of the possible potential binding sites and through further research it could be enhanced to improve the efficiency of docking with more accuracy and thereby inhibiting the protein in forming adhesion complexes and causing life-threatening infections.

Challenges and Limitations of Targeting P1 and P40/P90 Proteins for Mycoplasma pneumoniae Treatment:

While P1 and P40/P90 proteins hold promising potential as therapeutic targets for Mycoplasma pneumoniae infections, several challenges and limitations need to be addressed:

Adhesion Complexity:

P1 and P40/P90 mediate adhesion through complex interactions with multiple host cell receptors like vimentin and sialic acid. Targeting these interactions requires drugs that can disrupt multiple binding sites simultaneously, increasing development complexity.

Bacterial evasion mechanisms like phase variation and glycosylation can hinder drug efficacy by altering surface proteins and preventing binding. The binding site for sialic acid was found in P40/P90 and not in P1. Genetic and clinical variability concentrates on the N-terminal domain surfaces of P1 and P40/P90. Polyclonal antibodies

generated against the mostly conserved C-terminal domain of P1 inhibited adhesion of M. pneumoniae, and serology assays with sera from infected patients were positive when tested against this C-terminal domain. P40/P90 also showed strong reactivity against human infected sera. The architectural elements determined for P1 and P40/P90 open new possibilities in vaccine development against M. pneumoniae infections.

Specificity Concerns:

P1 and P40/P90 share structural and functional similarities with other bacterial adhesins. Developing drugs specific to M. pneumoniae while avoiding off-target effects on human proteins and other microbes is crucial.
Potential cross-reactivity with commensal bacteria could disrupt the delicate balance of the human microbiome and lead to unintended side effects.
Cross-reacting antigen
the membrane glycolipids of M. pneumoniae share a common antigen in the brain and lung tissues, which induce cross reaction. The carboxyl end of the P1 and P30 proteins in the adhesive organs of M. pneumoniae show high levels of homology to the cytoskeletal proteins, fibrinogen, keratin and troponin in eukaryotes . Thus, during infection, autoantibodies in the brain, lung, RBC-membrane, lymphocytes and myocardial cells commonly occur, which form immune complexes and magnify the autoimmune response, leading to multisystem immune damage.
Nutrition depletion
The small-genome M. pneumoniae does not possess the ability to self-synthesize amino acids, fatty acids, cofactors or vitamins. Instead, following permanent adherence via the adherend to the respiratory tract epithelia, M. pneumoniae spreads microtubules and inserts them into host cells, enabling oxygen consumption, use of glucose, absorption of cholesterol, ingestion of amino acids and consumption of nutrients in host cells, causing injury to the host cells

Resistance Development:

Bacteria are adept at developing resistance to antimicrobials. Overreliance on P1 and P40/P90 targeting drugs could lead to rapid resistance emergence, necessitating development of alternative strategies.
Combination therapies with other antibiotics or targeting different virulence factors might be necessary to prevent or delay resistance development. Most *Mycoplasma pneumoniae* infections are self-limiting; however, clinicians routinely treat pneumonia caused by *M. pneumoniae* with antibiotics. All mycoplasmas lack a cell wall and, therefore, all are inherently resistant to beta-lactam antibiotics (e.g., penicillin).

Delivery and Penetration:

Mycoplasma pneumoniae primarily resides within host cells, making it difficult for drugs to reach their target. Effective delivery systems that can penetrate the host cell membrane and reach the bacteria are needed.
Factors like biofilm formation and intracellular persistence further complicate drug delivery and require innovative approaches.

Vaccine Development:

Although P1 and P40/P90 are promising vaccine targets, their complex structures and potential for immune evasion pose challenges.
Development of effective vaccines requires a comprehensive understanding of immune response mechanisms against these proteins and the identification of conserved, immunodominant epitopes.

Immune damage

Animal experiments have shown that the histopathologic response occurs 10–14 days following primary M. pneumoniae infection, but within 3 days following secondary infection, indicating that the body responds via immune cell accumulation following M. pneumoniae infection, but produces a more marked immune response to a second infection (43). These findings indicate that the host immune response is important during the onset of M. pneumoniae-induced pneumonia.

Immunosuppression

M. pneumoniae infection can induce immunosuppression in the body and cause maladjustment of T cell subgroups. Experiments have revealed that M. pneumoniae infection causes severe destruction of B cells and T cells. At 13–18 weeks in patients infected with M. pneumoniae, the serum level of IgG declines. Certain children infected with M. pneumoniae suffer from hypoglobulinemia, decreased chemoattraction in neutrophils, lower reactivity to phytohemagglutin phytolectin and reduced resistance against combined infections with other pathogens, including S. pneumoniae. These changes indicate that M. pneumoniae infection may induce immunosuppression.

Developing multi-pronged therapeutic strategies targeting multiple adhesion pathways.
Designing specific inhibitors with high affinity and low off-target effects.
Investigating novel drug delivery systems for intracellular penetration.
Identifying conserved antigenic epitopes for vaccine development.
By addressing these challenges and leveraging the ongoing research, targeting P1 and P40/P90 proteins has the potential to significantly improve the prevention and treatment of Mycoplasma pneumoniae infections.

Significance of Research in Novel Therapeutic Strategies for Mycoplasma pneumoniae Infections:

Mycoplasma pneumoniae, a tiny bacterium lacking a cell wall, causes significant respiratory infections, particularly in children and young adults. Unfortunately, current treatment options are limited, often relying on macrolide antibiotics with potential side effects and increasing concerns about resistance. This highlights the crucial role of research in developing novel therapeutic strategies for Mycoplasma pneumoniae infections.

Addressing Antibiotic Resistance:

Mycoplasma pneumoniae exhibits concerning resistance to macrolides, tetracycline, or fluoroquinolone classes of antibiotics. Clinicians treat the disease with macrolide, tetracycline, or fluoroquinolone classes of antibiotics, taking age of the patient and local antibiotic resistance patterns into consideration:

- Macrolides (e.g., azithromycin): Children and adults
- Tetracyclines (e.g., doxycycline): Older children and adults
- Fluoroquinolones: Adults

Clinicians should not prescribe tetracyclines and fluoroquinolones for young children under normal circumstances. Macrolides are generally considered the treatment of choice. However, clinicians should practice prudent use of macrolide drugs due to the emergence of macrolide-resistant strains of *M. pneumoniae.*the current mainstay of treatment. Novel strategies aim to circumvent resistance mechanisms and provide effective alternatives.

This research can contribute to developing new classes of antibiotics with unique mechanisms of action, reducing reliance on existing antibiotics and potentially delaying resistance development.

Targeting New Vulnerabilities:

Research is exploring novel targets beyond the bacterial cell wall, including adhesion proteins like P1 and P40/P90, virulence factors, and metabolic pathways.
Targeting these vulnerabilities can offer more specific and effective treatment options, potentially reducing side effects and improving overall therapeutic outcomes.

Expanding Treatment Options:

Novel strategies can address the limitations of current treatments, such as poor penetration into host cells where Mycoplasma resides.
The development of targeted drug delivery systems and combination therapies can improve efficacy and broaden the therapeutic arsenal against this challenging pathogen.

Potential for Prevention:

Research into Mycoplasma pneumoniae virulence factors and immune response mechanisms could pave the way for vaccine development.
An effective vaccine could significantly reduce the burden of Mycoplasma pneumoniae infections, especially in vulnerable populations.

Improving Patient Outcomes:

By developing novel therapeutic strategies, researchers aim to improve treatment efficacy, reduce side effects, and shorten recovery times for Mycoplasma pneumoniae infections.
This can lead to improved patient quality of life and reduced healthcare costs associated with these infections.
Overall, research in novel therapeutic strategies for Mycoplasma pneumoniae infections holds immense significance for public health. It offers the potential to overcome current limitations, develop more effective treatments, and ultimately improve the lives of individuals affected by this challenging bacterial pathogen.

Referance:

Vimentin Is an Attachment Receptor for Mycoplasma pneumoniae P1 Protein
Kailan Peng,# a Yating Liao,# a Xia Li,# a Dongdong Zeng, b Youyuan Ye, a Li Chen, a Zhuo Zeng, a and Yanhua Zengcorresponding author John M. Atack, Griffith University. Published online 2023 Mar 13

Insights into the pathogenesis of Mycoplasma pneumoniae (Review)
Jun He Mihua Liu Zhufeng Ye Tianping Tan Xinghui Liu Xiaoxing You Yanhua Zeng Yimou Wu
Published online September 23, 2016

Immunodominant proteins P1 and P40/P90 from human pathogen Mycoplasma pneumoniae
David Vizarraga 1, Akihiro Kawamoto 2 3, U Matsumoto 4, Ramiro Illanes 1, Rosa Pérez-Luque 1, Jesús Martín 1, Rocco Mazzolini 5, Paula Bierge 6, Oscar Q Pich 6 7, Mateu Espasa 8, Isabel Sanfeliu 8, Juliana Esperalba 9, Miguel Fernández-Huerta 9, Margot P Scheffer 10, Jaume Pinyol 7, Achilleas S Frangakis 10, Maria Lluch-Senar 5, Shigetarou Mori 11, Keigo Shibayama 11, Tsuyoshi Kenri 11, Takayuki Kato 2 3, Keiichi Namba 2 12 13, Ignacio Fita 1, Makoto Miyata 14 15, David Aparicio 16. Published online 2020 Oct 14

Novel aspects on the pathogenesis of Mycoplasma pneumoniae pneumonia and therapeutic implications
Takeshi Saraya,1 Daisuke Kurai,1 Kazuhide Nakagaki,2 Yoshiko Sasaki,3 Shoichi Niwa,3 Hiroyuki Tsukagoshi,3 Hiroki Nunokawa,1 Kosuke Ohkuma,1 Naoki Tsujimoto,1 Susumu Hirao,1 Hiroo Wada,1 Haruyuki Ishii,1 Koh Nakata,4 Hirokazu Kimura,5 Kunihisa Kozawa,3 Hajime Takizawa,1 and Hajime Goto1.Published online 2014 Aug 11