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Original Article

Shikimate Kinase 1 from *Klebsiella pneumoniae* as a New Drug Target Enzyme: Insights from Comparative Modeling and Molecular Dynamics

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ABSTRACT

Background: *Klebsiella pneumoniae*, a Gram-negative bacterium, is an established nosocomial pathogen that is particularly dangerous to immunosuppressed individuals, causing diseases such as sepsis, pneumonia, urinary tract infections and respiratory tract infections. The emergence of multidrug resistant strains of this bacterium poses a significant challenge to current therapeutic strategies, highlighting the urgent need for new drug targets. **Materials and Methods:** Our study highlights the shikimate pathway as a source of such targets, given its role in the production of essential aromatic compounds in various organisms, but its absence in humans. We focus on Shikimate Kinase 1 (SK-1), encoded by the *aroK* gene in *K. pneumoniae* and use it as a model for inhibitor development. Through comparative modelling, structural validation is using Ramachandran plots, ERRAT and Verify3D and stability checks using energy minimization and molecular dynamics simulations. **Results:** In the Ramachandran plot validation, AlphaFold2 performed better than the other three predicted models (SWISS-MODEL, Phyre2 servers, I-TASSER). The Ramachandran plot analysis showed 90.8% residues in the preferred region, 8.8% residues in the allowed region. When Desmond's energy minimization calculations were applied to the models, AlphaFold2 showed the lowest energy. In addition, MD simulations representing a stable conformation were applied to the reduced structure. **Conclusion:** This provides a basis for molecular coupling and the exploration of new inhibitors, offering promising avenues for the development of treatments against *K. pneumoniae*.

Keywords: *Klebsiella pneumoniae*, Shikimate Kinase 1, Comparative modeling, AlphaFold2, Molecular dynamics simulations.

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INTRODUCTION

Klebsiella pneumoniae, a Gram-negative bacterium, is considered an important nosocomial pathogen and contributes to a variety of diseases, particularly in immunocompromised patients.[1] In particular, it is the main cause of diseases such as septicemia, pneumonia and infections of the respiratory and urinary tracts.[2] The increasing prevalence of multidrug resistant strains of *K. pneumoniae* poses a significant challenge in the treatment of this pathogen and increases the complexity of therapeutic interventions. This situation poses an enormous threat to public health and highlights the urgent need for the discovery of new drug targets.

Among the potential targets for drug development against bacterial diseases, the shikimate pathway deserves special attention. In plants and microorganisms, all major aromatic compounds involved in primary metabolism are produced by the Shikimate Pathway (SP).[3] It is a seven-step biosynthetic pathway that generates chorismic acid (the major branching point for the synthesis of aromatic amino acids, ubiquinone and secondary metabolites) from phosphoenolpyruvate and erythrose-4-phosphate.[4] This pathway is essential for algae, higher plants, bacteria, fungi and apicomplexan parasites, but is absent in humans.[5] Therefore, SP enzymes are potential targets for the development of non-toxic antibacterial drugs and herbicides.[6]

Our research focuses on Shikimate Kinase 1 (SK-1), the fifth enzyme in SP. This enzyme mediates the phosphorylation of the 3-hydroxyl group of shikimate using ATP to produce Shikimate 3-Phosphate (S3P) and ADP. Notably, the *aroK* gene encoding SK-1 has been identified in *K. pneumoniae*, but it is absent in humans and was therefore selected as a target for inhibitor design.

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Comparative modeling and structural validation for KpSK-1 were performed using Ramachandran plot analysis, ERRAT, Verify3D alongside energy minimization and molecular dynamics simulations to confirm structural stability. Our findings shed light on a likely universal mechanism at the active site, paving the way for molecular coupling studies and the discovery of novel inhibitors, potentially heralding a new class of therapeutics for *K. pneumoniae* infections.

MATERIALS AND METHODS

Retrieval of Sequences

Amino acid sequences of SK-1 (*aroK*) from *K. pneumoniae* (accession numbers: A0A0H3GK99) were retrieved from the UniProt database (https://www.uniprot.org/). This protein has 177 amino acids. It is encoded by the *aroK* gene.

Analysis of primary structures

Protein primary structure analyzes were performed using the Expasy ProtParam tool (https://web.expasy.org/protparam/).[7] ProtParam was used to compute a range of physical and chemical characteristics of the protein. Key attributes comprised Molecular Weight (MW), net charge, theoretical Isoelectric point (pI), extinction coefficient, estimated half-life, instability index, aliphatic index and Grand Average hydropathy (GRAVY). These properties help isolate and purify the protein. The amino acid sequence of KpSK-1 was used as input.

Prediction of secondary structures

PSI-BLAST Predicted the Secondary structure of KpSK-1 based on secondary structure Preditcion (PSIPRED) V4.0 (http://bioinf.cs.ucl.ac.uk/psipred/), Self-Optimized Prediction method with Alignment (SOPMA),[7] Chou and Fasman Secondary Structure Prediction Server (CFSSP).[8]

Modeling of protein structure

Currently, the experimental structure of KpSK-1 is not listed in the Protein Data Bank (PDB). Homology modeling was used to predict the 3D structure. The 3D structure of KpSK-1 was estimated using the following methods. SWISS-MODEL,[9] Phyre2, I-TASSER[10] and the AI-based program AlphaFold2 Server.[11]

Energy Minimization of Predicted Structure

3D protein models were sent to the Desmond module for energy minimization. Using its own optimized force field, Desmond performs energy minimization on 3D protein models.[12]

Validation of 3D models

To validate the predicted protein structures, a Ramachandran plot analysis was generated using SAVES v6.0[13] taking into account parameters such as preferred, allowed and freely allowed regions of amino acid residues. A Ramachandran plot was generated by uploading PDB files of the SWISS-MODEL, Phyre2, I-TASSER and AlphaFold2 models for the target genes. These were generated for the predicted models and the most stable and effective model was determined by comparative analysis. The validation process was improved by using the ERRAT and Verify3D tools.[14,15] ERRAT examines the statistics of nonbonded interactions between different types of atoms and plots the value of the error function against a 9-residue sliding window position determined by comparing the data with the statistics of highly refined structures. Simultaneous

Verify 3D determines whether a 3D atomic model and its corresponding 1D amino acid sequence are compatible by classifying the atoms according to their position and environment (alpha, beta, loop, polar, nonpolar, etc.,) and comparing the results with established frameworks.

Molecular Dynamics Simulation

An MD simulation was performed based on the model with the lowest energy. The iMODS server (http://imods.chaconlab.org/) was used to find the optimal model stability.[16] Using Normal Mode Analysis (NMA) to generate the internal coordinates of the protein, the server assesses the stability of the protein. Stability was described using the eigenvalue, variance, covariance matrix and elastic network model parameters.

RESULTS

Analysis of Protein Primary Structures

The Expasy ProtParam tool was used to predict various physicochemical properties of our protein. In particular, Molecular Weight (MW) and Isoelectric point (pI) are important parameters in protein purification. There are twenty negatively charged amino acid residues (Asp+Glu) and nine positively charged amino acid residues (Arg+Lys) in the protein. The purity of the protein sample can be estimated from the extinction coefficient. After calculating the instability index for the *in vitro* stability assessment, the stability of the protein was reported to be 20.16. KpSK-1 has a high aliphatic index of 91.53, indicating thermostability. An aliphatic index measures the area occupied by aliphatic side chains. KpSK-1 is hydrophilic, as indicated by its Globular Protein Hydrophobicity (GRAVY) value of -0.144, which indicates hydrophobicity or hydrophilicity. A brief summary of the properties of KpSK-1 determined by ProtParam is given in Table 1.

Prediction of Secondary Structures

The secondary structure of KpSK-1 was predicted using PSIPRED, SOPMA and CFSSP. These provided information on the secondary structures of the target KpSK-1, such as α-helix, β-helix and helix. PSIPRED is a protein structure prediction system that allows us to input a protein sequence, make a prediction and obtain the results in graphical form. PSIPRED analysis showed that KpSK-1 has 99

α-helices, 57 coiled coil regions and 21 elongated chains, with a high degree of confidence in most regions. Supplementary Figure 1a shows the α-helices, elongated chains and coil regions predicted by PSIPRED. SOPMA is a protein structure prediction server that uses consensus predictions derived from multiple alignments. This approach has been instrumental in the advancement of our understanding of the secondary structure of protein. According to the SOPMA analysis, KpSK-1 had 91 α-helices, 21 extended helices, 18 β-helices and 47 random helices. Supplementary Figure 1b shows the α-helices, β-helices, β-helices and random helices predicted by SOPMA. The CFSSP method uses the Chou and Fasman algorithm to predict secondary protein structures. According to the CFSSP analysis, KpSK-1 had 143 α-relics, 66 β-sheets (extended chains), 24 β-loops and 8 random helices. Supplementary Figure 1c shows the α-helices, β-sheets, β-loops and random helices predicted by CFSSP. Table 2 shows the display of secondary structure information from different servers.

Table 1: Prediction of physicochemical properties of KpSK-1 by the Expasy Prot Param tool.

Property	Shikimate kinase 1
Sequence ID	A0A0H3GK99
Molecular weight	19.400.14
Number of amino acids	177
positively/negatively charged number	19/20
Theoretical pI	6.59
A280 molar extinction coefficient (M^1 cm^1)	9.970 (cysteine reduced)
A280 molar extinction coefficient (M^1 cm^1)	10.095 (cystine bridges)
Estimated $t_{1/2}$	30 hr
Instability index	20.16 (stable)
Aliphatic index	91.53
GRAVY	-0.144

Modeling of Protein Structure

There is currently no experimental structure for KpSK-1 in the Protein Data Bank (PDB). Homology modelling was used to predict its 3D structure. SWISS-MODEL, Phyre2, I-TASSER and the AI-based AlphaFold2 server were used. The Swiss-MODEL platform facilitates automated three-dimensional comparative modelling of protein structures for researchers around the world. Phyre2, a web-based service, predicts protein structure, function and mutations. I-TASSER uses threading calculations and iterative structure discovery simulations to identify optimal fragments and generate sequence template alignments. Numerous models were generated and the best model was selected based on z-scores. We extensively searched the Protein Data Bank (PDB) to identify sequences that might correlate with experimental structures. AlphaFold2, which uses a novel machine learning approach, integrates physical and biological knowledge into the deep learning algorithm through multiple sequence alignments. AlphaFold2 stands out as the first computational method to consistently deliver protein structures with atomic precision, even in cases where the template is unknown. It outperforms other methods, often achieving accuracy comparable to that of experimental structures.

Protein Structure Modeling with SWISS-MODEL

The target 3D protein structure was constructed using the fully automated SWISS-MODEL server. First, the homology modelling of the target protein was compared with a similar protein structure template. For KpSK-1, SWISS-MODEL generated 50 templates and the optimal model, identified by alignment coverage and Z-score considerations, is shown as 1e6c (Figure 1a).

Protein Structure Modeling Using Phyre2 Server

Using the Phyre2 server, we predicted 3D models and identified ligand binding sites. Of the 120 templates obtained from the Phyre2 server, 20 were associated with models, while the remaining templates were not modelled. The selection of the best modelled template was based on maximum coverage, represented by d1e6ca (Figure 1b).

Table 2: Information on secondary structures represented by different servers.

Position in Sequence	PSIPRED	SOPMA	CFSSP
1-20	coils	coils	extended strands
20-40	α-helix	α-helix	α-helix
40-60	α-helix	α-helix	α-helix
60-80	α-helix	α-helix	extended strands
80-100	α-helix	extended strands	α-helix
100-120	α-helix	coils	α-helix
120-140	α-helix	α-helix	α-helix
140-160	α-helix	α-helix	α-helix
160-177	α-helix	α-helix	α-helix

Protein Structure Modeling Using I-TASSER

The I-TASSER server predicted 10 models, from which the demonstration with the best C score of 0.61 (3trfA) with an estimated precision of 0.80 (TM score) and 3.9 (RMSD) (Figure 1c) was selected. The C-score is a confidence score for estimating the quality of predicted models by I-TASSER. It is calculated on the basis of the significance of the threading template alignments and the convergence parameters of the structure assembly simulations.

Protein Structure Modeling Using AlphaFold2 Server

3D structure of KpSK-1 generated by AlphaFold2 (Figure 1d). Most residues in the developed model have high confidence scores that correlate with model accuracy (pLDDT>90, Supplementary Figure 2). AlphaFold2 generates a per residue confidence score (pLDDT) between 0 and 100 in the model, the region below 50 pLDDT scores revealed the unstructured region.

Structure Validation

Based on the best predicted models from SWISS-MODELL, Phyre2, I-TASSER and AlphaFold2, an online database called SAVES v6.0 was used to generate Ramachandran plot analysis, ERRAT and VERIFY 3D to explore the 3D models. In the Ramachandran plot, amino acids (residues) were distributed in three regions. These three different regions were preferred, allowed and liberally allowed regions. The overall quality scores of ERRAT are above 90%. Approximately 79.66% of the predicted protein residues (AlphaFold2) have an average 3D-1D score>=0.1. The results of the Ramachandran plot analysis, ERRAT and VERIFY 3D are shown in Table 3 and Supplementary Figures 3-6.

Energy minimization

Desmond performs energy minimization on 3D protein models using its proprietary optimized force field. The comparison of energy minimization values identifies AlphaFold2 as the least energetic model with an energy value of E= -550,600 kcal/mol.

Molecular Dynamics Simulation

After comparing the energy minimization results, a further analysis was performed using MD simulation on the better predicted protein model (AlphaFold2) which had the lowest minimization value. In this study, the Normal Mode Analysis (NMA) of the iMODS web server was used to assess physical activity and stability. NMA is a computational approach to study and characterize protein flexibility.[17] Although working with very large macromolecules, iMODS facilitated the study of different modes and generated logical pathways for the transition between two similar structures.[18] With the preservation of implicit stereochemistry, the unique internal coordinate formulation broadens the applicability of NMA and improves its efficiency.

Following comparison of energy minimization results, the predicted protein model (AlphaFold2) with the lowest

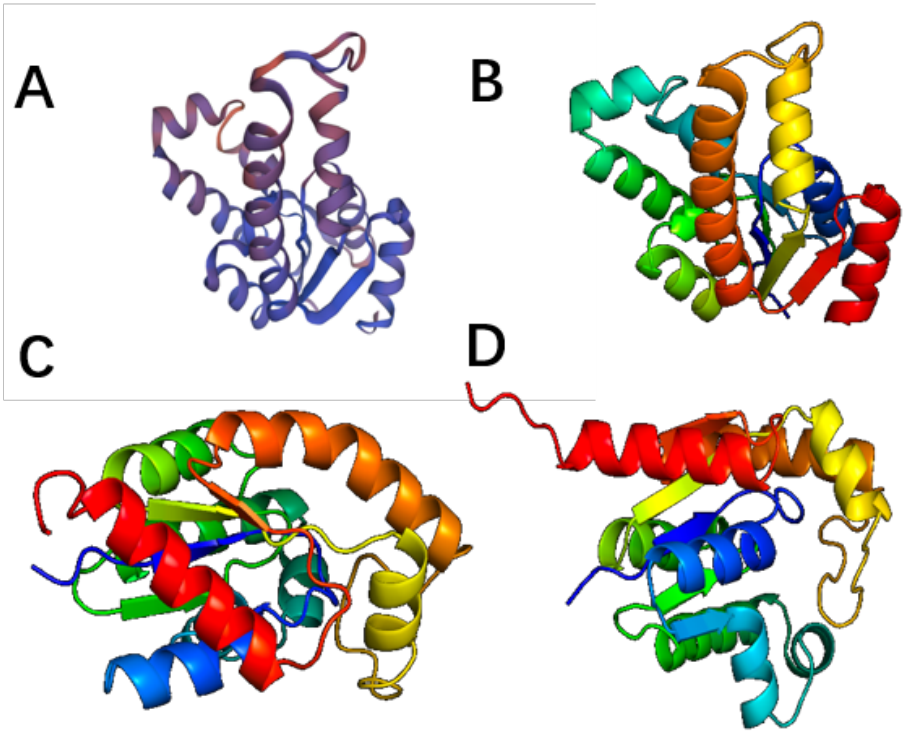

Figure 1: Predicted models for SWISS-MODEL (A), Phyre2 (B), I-TASSER (C) and AlphaFold2 (D).

Table 3: Comparison of different KpSK-1 models using the Ramachandran plot, ERRAT and VERIFY 3D.

Properties	PROCHECK								ERRAT	Verify3D
	Ramachandran Plot- Allowed region	Additionally allowed	Generously allowed.	Disallowed region.	Number of non-glycine and non-proline residues	Number of end-residues	Number of glycine residues	Number of proline residues	Total number of residues	
SWISS-MODEL	138(92.6%)	10(6.7%)	0(0.0%)	1(0.7%)	149	2	12	7	169	99.37%	57.06%
Phyre2	138(93.2%)	9(6.1%)	0(0.0%)	1(0.7%)	153	2	12	7	177	99.38%	63.31%
I-TASSER	125(80.6%)	23(14.8%)	6(3.9%)	1(0.6%)	155	2	12	7	177	99.41%	64.41%
AlphaFold2	145(93.5%)	8(5.2%)	2(1.3%)	0(0.0%)	155	2	12	7	177	90.48%	79.66%

minimization value was further analyzed by MD simulation. Using the NMA feature of the iMODS web server, stability and physical activities were evaluated in this study. When dealing with large molecules, iMODS allows exploration of different modes and generation of reliable transition paths between homologous molecules. The unique internal coordinate formulation not only improves the efficiency of NMA but also broadens its applicability, while implicitly preserving stereochemistry.

The rigidity of the motion is characterized by the eigenvalue corresponding to each type of vibration. The energy required to change the structure determines its magnitude.[19] A lower eigenvalue means the easier deformation. For the predicted 3D structure, the calculated eigenvalue was 1.103e-04 (Figure 2a). The eigenvalue and the variation associated with each mode have an inverse relationship. The colored bar chart illustrates the presence of individual (purple) and cumulative (green) variances (Figure 2b). The covariance matrix indicates coupling between pairs of residues, i.e. whether they experience correlated (red), uncorrelated (white) or anti-correlated (blue) motions (Figure 2c). To simulate spring-connected atoms connected by springs,[20] an elastic network model was formulated. In the corresponding diagram, each dot represents a spring connecting a pair of atoms, with darker grays indicating stiffer springs.[21] These dots together represent improved stability (Figure 2d).

DISCUSSION

KpSK-1, which is located between chorismate synthase and 5-enolpyruvylshikimate-3-phosphate, plays a crucial role in the pathway, particularly in the fifth step, the conversion of shikimate to shikimate-3-phosphate using ATP as a co-substrate[4]. Because of the structural homology of their substrates, it is conceivable that multitargeted antibacterial drugs could be developed that target multiple enzymes in this cascade. Such an approach could significantly reduce the risk of resistance development.

As the full 3D structure of KpSK-1 is not available in the Protein Data Bank (PDB), a homology modelling strategy was used to generate the full structure using a variety of tools. An important development in computational biology is homology modelling, in which the three-dimensional structure of a target protein is determined by comparing the sequences of the template and the target. This approach is particularly useful for difficult cases, such as viral proteins that do not crystallize, as it improves our understanding of receptor-ligand interactions.

AlphaFold2, Phyre2 server, I-TASSER, SWISS-MODEL and Phyre2 participated in the prediction of the KpSK-1 structure. KpSK-1 structure prediction. Each study must include validation. A Ramachandran plot was used to check the main model and the results showed that 93.5% were within the preferred region. The best model was then determined by energy minimization, which is an important parameter in assessing stability. Reduced energy values indicate increased stability, so the AlphaFold2 model with

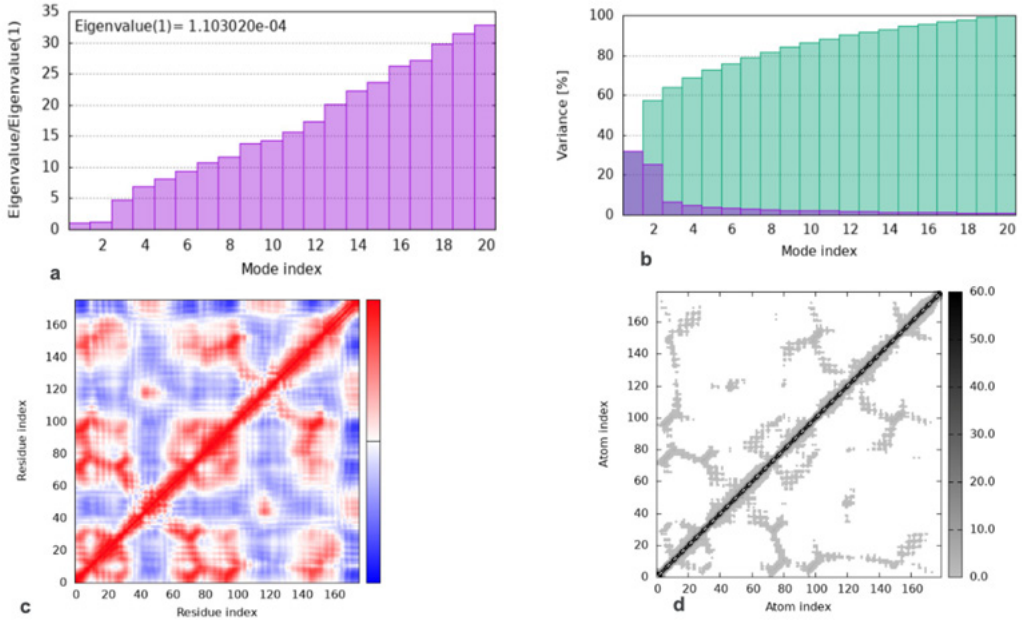

Figure 2: Molecular Dynamics Simulation of AlphaFold2 predicted 3D structure of KpSK-1 with iMODS server (a) Eigenvalue (b) Variance (c) Covariance matrix (d) Elastic network.

the lowest energy (-550,600 kcal/mol) was selected for further study. The results of subsequent AlphaFold2 MD simulations showed increased stability. Further studies may investigate the active sites of the target protein, opening the door to molecular modeling studies to identify suitable substrates for KpSK-1. For many years, the ability to predict three-dimensional protein structures in the absence of experimental data has been largely dependent on computational techniques. A notable development in this area is AlphaFold2, a groundbreaking application of deep learning algorithms to biomedicine that has drastically changed the field of structural biology.[22] With remarkable accuracy and speed, AlphaFold2 can take both a protein sequence and a template structure as input. It is a major step forward, as it can predict the structure of around 400 residues in less than a minute on a single GPU[11]. The process involves retrieving the Protein Data Bank (PDB) sequence of the target protein and using a Colab notebook to predict the 3D structure based on the given sequence.

CONCLUSION

The current preliminary investigation aimed to understand the structures of KpSK-1 using a variety of in silico techniques and methodologies. In Ramachandran plot validation, AlphaFold2 performed better than the four predicted models (SWISS-MODEL, Phyre2 servers, I-TASSER and AlphaFold2). Ramachandran plot analysis showed 90.8% residues in the preferred region, 8.8% residues in the allowed region and 0.4% residue in the generously allowed region. When Desmond's energy minimization calculations were applied to the models, AlphaFold2 showed the lowest energy (E=-550,600 kcal/mol). In addition, MD simulations representing a stable conformation were applied to the reduced structure. This work has provided molecular insight for future studies to determine the structural and functional properties of KpSK-1 to find or develop a new drug target enzyme using a structure-based drug design approach.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

Conceptualization: Suresh Kumar. Data curation: Yanping Li. Formal analysis: Yanping Li and Lihu Zhang. Methodology: Suresh Kumar and Lihu Zhang. Writing - original draft: Yanping. Writing - review and editing: Yanping Li.

ABBREVIATIONS

SK-1: Shikimate kinase 1; **SP:** Shikimate pathway; **S3P:** Shikimate 3-phosphate; **MW:** Molecular weight; **pI:** Isoelectric point; **NMA:** Normal Mode Analysis; **pLDDT:** Prediction value of local distance difference test.

SUMMARY

This study highlights the shikimate pathway, specifically shikimate kinase 1 (SK-1) in *K. pneumoniae*, as a potential drug target due to its absence in humans. AlphaFold2 outperformed other models in predicting the structure of SK-1, validating its

accuracy and stability through Ramachandran plots, energy minimization and MD simulations. This provides a basis for molecular targeting and inhibitor development, offering hope for new therapeutic strategies against multidrug-resistant *K. pneumoniae*.

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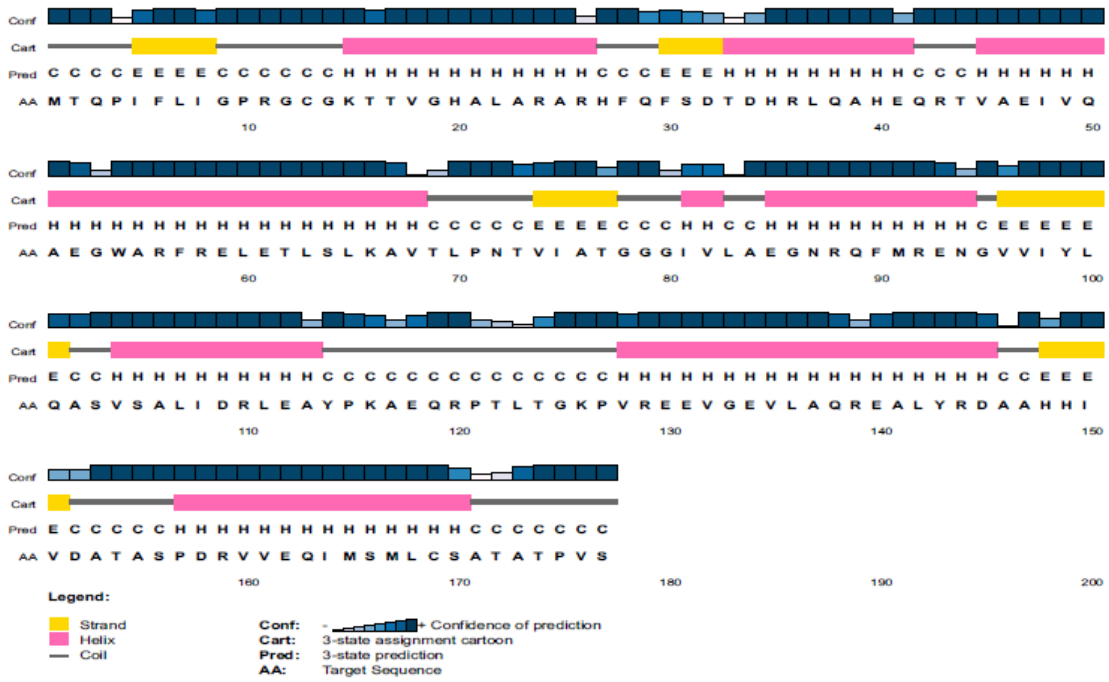
Supplementary 1 (Prediction of secondary structures)



Supplementary Figure 1a: Depicts the α-helices, extended strands, and coil regions predicted by PSIPRED.

Supplementary Figure 1b: Depicts the α-helices, β-strands, β-turns and random coils predicted by SOPMA.

Target Sequence:

```
         10         20         30         40         50         60         70
MTQPIFLIGP RGCGKTTVGH ALARARHFQF SDTDHRLQAH EQRTVAEIVQ AEGWARFREL ETLSLKAVTL

         80         90        100        110        120        130        140
PNTVIATGGG IVLAEGNRQF MRENGVVIYL QASVSALIDR LEAYPKAEQR PTLTGKPVRE EVGEVLAQRE

        150        160        170
ALYRDAAHHI VDATASPDRV VEQIMSMLCS ATATPVS
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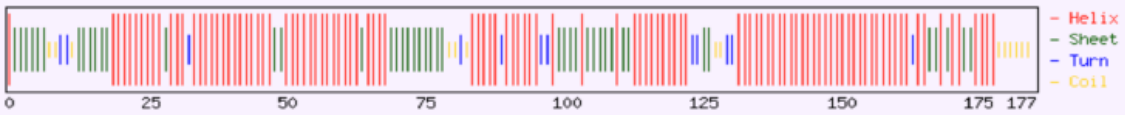


Secondary Structure:

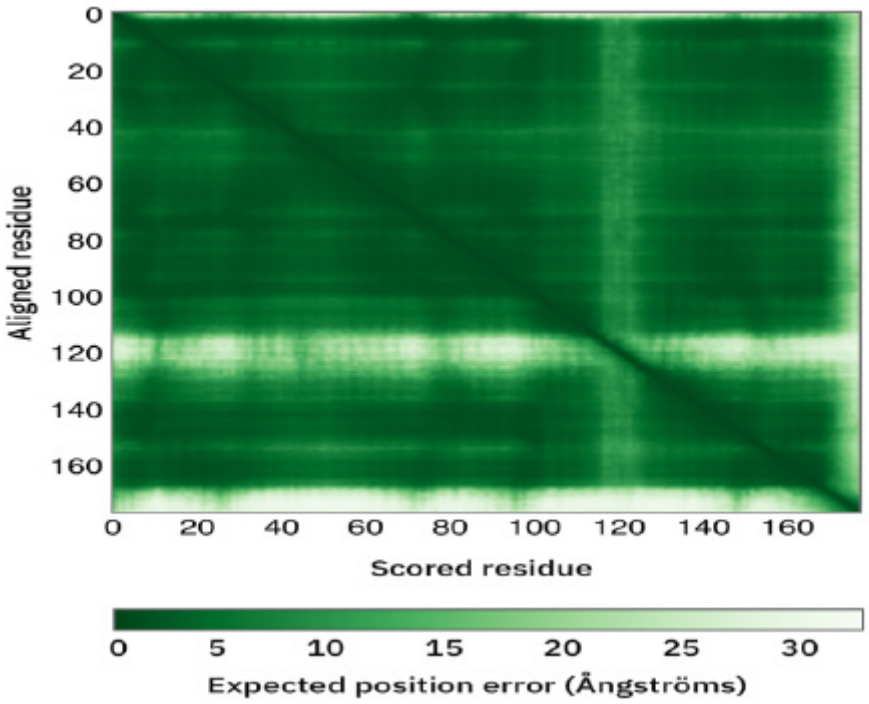
```
                  *         *         *         *         *         *
Query 1    MTQPIFLIGPRGCGKTTVGHALARARHFQFSDTDHRLQAHEQRTVAEIVQAEGWARFRELETLSLKAVTL 70
Helix 1    HHHHHHH           HHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHH  70
Sheet 1    EEEEEEE     EEEEEE          EE                EEEEEE         EEEEEEEEEEEEE 70
Turns 1      T       TT                        T          T          T               70
Struc 1    HEEEEEECCTTCEEEEEEHHHHHHHHHEHHHTHHHHHHHHHHHHHHHEEHHHHHHHHHHHHHEHHHHEEEE 70

                  *         *         *         *         *         *
Query 71   PNTVIATGGGIVLAEGNRQFMRENGVVIYLQASVSALIDRLEAYPKAEQRPTLTGKPVREEVGEVLAQRE 140
Helix 71             HHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHH        HHHHHHHHHHHHHHH 140
Sheet 71   EEEEEE                      EEEEEEEEEEEEE           EEEE                 140
Turns 71    T       T       T T     TT        T             T  TTT    TT    T         T 140
Struc 71   EEEEEECCTCHHHHHTHHHHHHTTHEEEEHEEEEEHEEHHHHHHHHHHTTEECCTTHHHHHHHHHHHHHHH 140

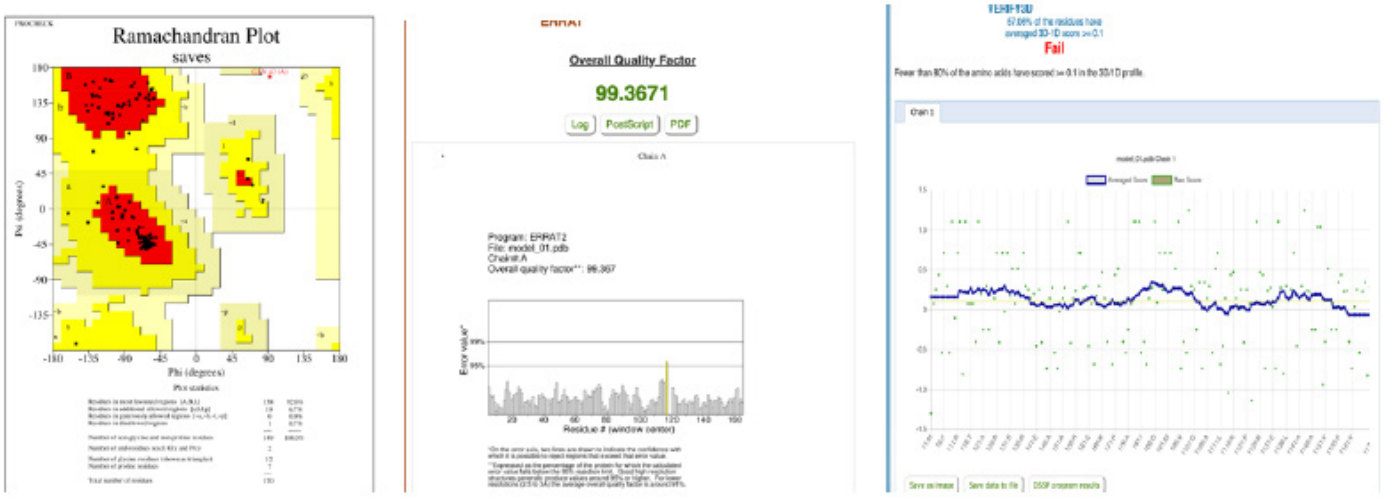
                  *         *         *
Query 141  ALYRDAAHHIVDATASPDRVVEQIMSMLCSATATPVS 177
Helix 141  HHHHHHHHHHHHHHHHHHHHHHHHHHHHHHH       177
Sheet 141                    EEEEEEEEE           177
Turns 141     T            TT                    177
Struc 141  HHHHHHHHHHHHHHHHHTHHEEHEHHEEHHHHCCCCCC 177


Total Residues: H: 143   E: 66    T: 24
       Percent: H: 80.8  E: 37.3  T: 13.6
```

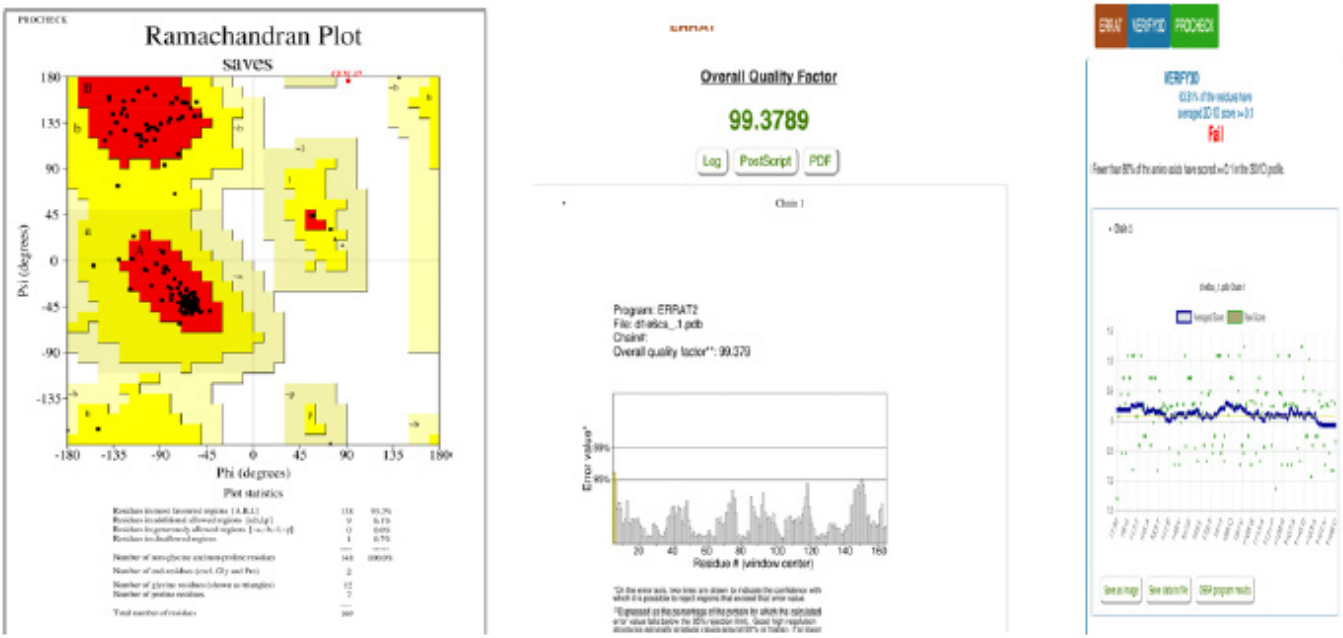
Supplementary Figure 1c: Depicts the α-helices, β-β-sheets, β-turns and random coils predicted by CFSSP.



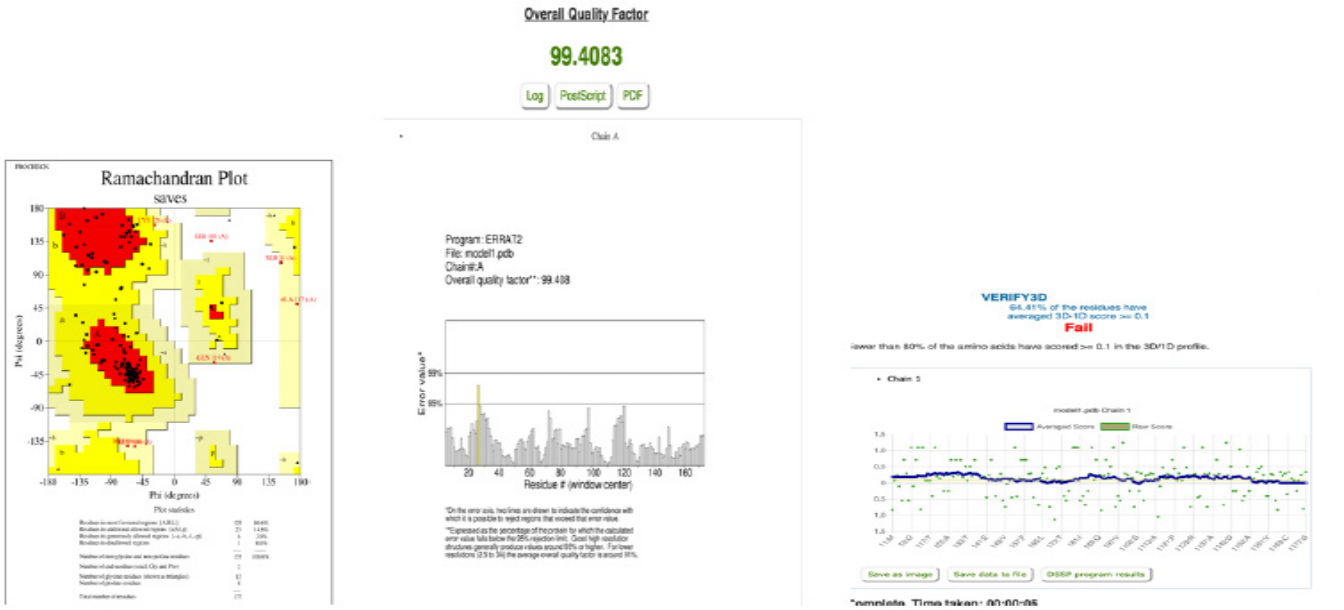
Supplementary Figure 2a: pLDDT of AlphaFold2



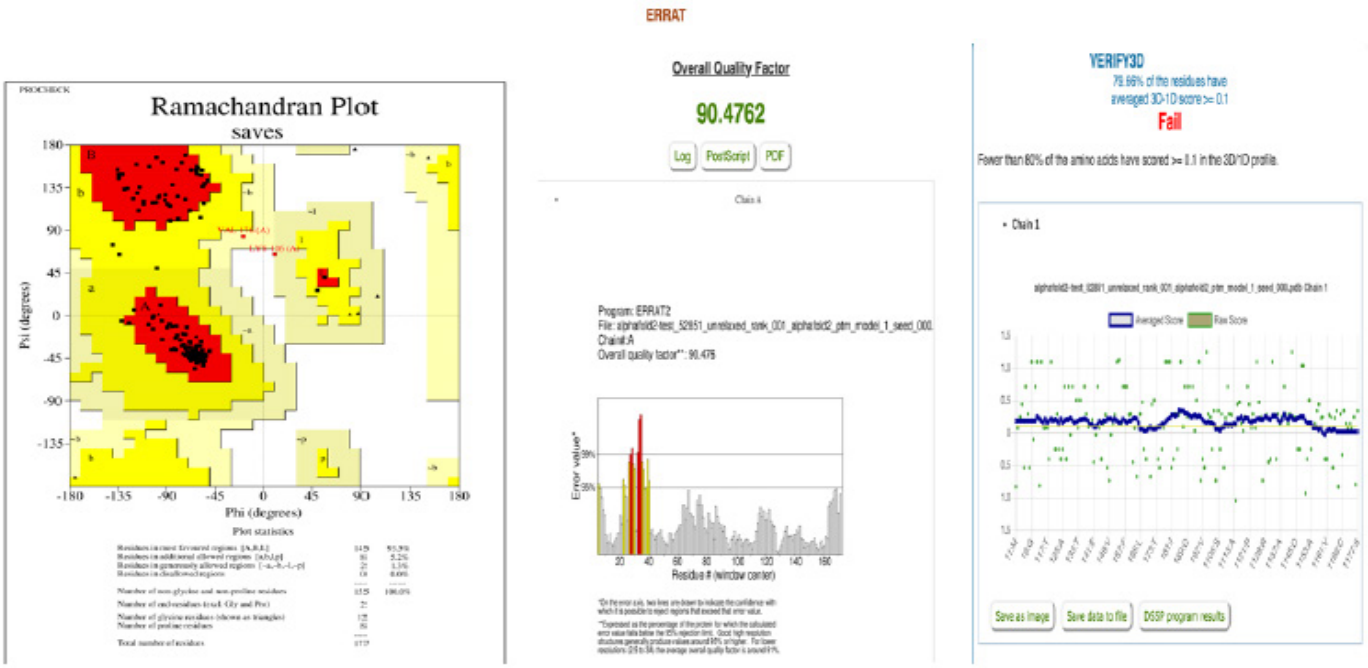
Supplementary Figure 3: SWISS-MODELL.



Supplementary Figure 4: Phyre2.



Supplementary Figure 5: I-TASSER.



Supplementary Figure 6: AlphaFold 2.