IN SILICO INSIGHTS INTO CLEC4G, TD26, AND NRF2 AS THERAPEUTIC TARGETS IN HEPATOCELLULAR CARCINOMA

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Abstract

Hepatocellular carcinoma is the 6th most occurring cancer in the world it causes approximately 8,30,000 deaths every year making it one of the leading reasons for cancer related deaths. In this project, we performed molecular docking to investigate the binding affinities of anti-HCC agents with three selected proteins: C-type lectin domain family 4 member G (CLEC4G), TD26, and Nuclear factor erythroid 2-related factor 2 (NRF2). CLEC4G is involved in immune modulation within the liver, TD26 is involved in tumor progression, and NRF2 plays a crucial role in cellular defense mechanisms. Using HDock and PyMOL, we performed molecular docking simulations to evaluate interactions between selected anticancer agents like Sorafenib, Lenvatinib, Regororafenib, Cabozantinib, Doxorubicin with these proteins. The selected ligands showed different binding affinities wherein Doxorubicin (-153.87, -137.42 and -122.77) showed the strongest binding affinity with all the three proteins followed by Cabozantinib, Regorafenib, Lenvatinib and sorafenib respectively. Our findings focus on the therapeutic potential of targeting CLEC4G, TD26, and NRF2 in HCC treatment. Additional lab experiments are needed to verify these results and study their Practical applications. This study provides valuable insights into the development of targeted therapies for HCC, paving the way for precision medicine approaches to improve patient outcomes.

Keywords: *Hepatocellular carcinoma, cancer, CLEC4G, NRF2, TD26, Molecular docking*

1. Introduction

The liver is a vital organ located in the upper right part of the stomach, below the diaphragm. It is the largest internal organ and has various important functions that are crucial for maintaining overall health. Hepatocellular carcinoma (HCC) is the sixth most common type of liver cancer, ranking 3rd in cancer-related deaths (Schlageter *et al.*, 2014), arising from the hepatocytes, which are the main functional and structural units of the liver. It is a major global concern due to its high death rate and increase in incidence. HCC usually develops in individuals with chronic liver disease (Chu, C. M., 2000), Viral infections like Hepatitis B and Hepatitis C cause inflammation in the liver cells (Boltjes, A *et al.*, 2014), which

in turn get damaged, increasing the risk of cirrhosis and HCC, other causes may include overconsumption of alcohol and cigarette smoking.

HCC is characterized by atypical hepatocytes with pleomorphic nuclei and abnormal mitoses. In HCC, angiogenesis is a key factor in tumor progression; the tumor appears as single or multiple nodules. The tumor may grow in surrounding liver tissue or metastasize to distant organs, particularly the lungs and lymph nodes, in advanced stages.

In the early stages, HCC may be asymptomatic, especially when the tumor is small or confined to a single area of the liver, but after the cancer progresses, it shows symptoms like loss of appetite, loss of weight, yellowing of skin and eyes, swelling and fluid accumulation in the abdomen, enlargement of the liver, nausea, and vomiting.

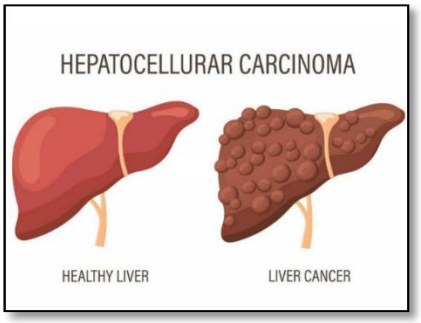


Figure 1.1 Hepatocellular Carcinoma

Chronic liver injury, immune evasion, and genetic mutations are the reasons for the development of HCC. Major factors include HBV, HCV, non-alcoholic liver diseases, and non-alcoholic fatty liver; these may cause oxidative stress that enhances DNA damage, long-term inflammation, and fibrosis. The tumor microenvironment is created due to cirrhosis, which promotes hepatocyte regeneration and genetic instability, it is highly immunosuppressive and characterized by overexpression of programmed death ligand 1 (PD-L1), helping the tumor cells escape immune surveillance (Hao *et al.*, 2023). Certain molecular events in cancer cells due to abnormal of overexpression of a few proteins result in aggressive tumor progression and therapy resistance.

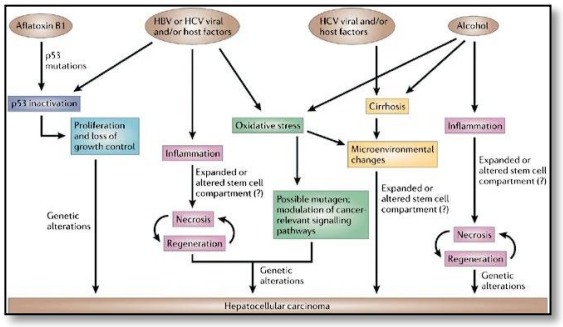


Figure 1.2 Pathogenesis of Hepatocellular Carcinoma

Proteins play a crucial role in cancer by regulating cell growth, division, and survival. Oncogenic proteins, such as mutated growth factors, kinases, and transcription factors, drive uncontrolled proliferation by activating signaling pathways. Moreover, proteins involved in apoptosis, DNA repair, and immune regulation impact tumor development and response to therapy due to their inactivation or abnormal functioning. These dysregulated protein expressions or functions can promote metastasis, drug resistance, and immune evasion, making proteins key targets for cancer diagnosis and treatment.

Clec4g (C-type lectin domain family 4 member G) is a liver-specific receptor mainly expressed in liver sinusoidal endothelial cells. It plays a role in immune regulation by altering antigen recognition and immune cell activation. In hepatocellular carcinoma (HCC), Clec4g has been involved with tumor immune escape and metastasis by interacting with immune checkpoints and inflammatory pathways. Its abnormal expression in HCC patients suggests its role in tumor progression and immune suppression.

Nuclear factor erythroid 2-related factor 2 (NRF2) is a key transcription factor that regulates antioxidant responses and cellular defense mechanisms (He *et al.,* 2020) It generally protects the normal cells from damage caused by reactive oxygen species. In HCC, NRF2 activation helps cancer cells survive oxidative stress and gives resistance to chemotherapy. Thus, NRF2 has a dual role, acting as both a protector in normal liver function and a driver of malignancy in HCC.

Hepatocellular carcinoma-associated protein TD26, also known as betatrophin or ANGPTL8, is a protein predominantly expressed in the liver. Research indicates that TD26 is overexpressed in hepatocellular carcinoma (HCC) tissues compared to normal liver tissues. Clinically, elevated TD26 levels has been correlated with larger tumor sizes (Wang *et al.,* 2018). and poorer overall survival.

2. Methodology

2.1 Homology Modelling

Target protein sequences were retrieved using NCBI, PDB, Selection criteria included selecting proteins with amino acid sequences less than 600 without a 3D image in NCBI..Clustal omega and BLAST were performed to compare the target sequence with template protein sequences. Click on run BLAST present at top right under 'Analyze this sequence', select BLASTp to compare the query sequence with template protein sequences, template sequences with maximum HIIT score were selected, FASTA of the template proteins were retrieved and saved.

2.2 Model Generation and Validation

SWISS-MODEL which is a web-based tool was used to generate a 3D protein model based on sequence similarity. The protein was submitted in FASTA format to generate the model

2.3 Protein Validation

Saves v6.0 which is a toolkit that includes protein validation parameters like ERRATT, VERIFY3D, PROCHECK and WHAT CHECK to check the protein quality.PROSA was used to check the errors of the model generated. After modelling of the protein, it was saved in PDB format and was uploaded in Saves v6.0 and ProSa for the validation.

2.4 Active Site Prediction

Active sites of the proteins generated proteins were done using CASTpfold, a tool used to find active pockets and topological properties of the protein structure

2.5 Selection of Ligands

For molecular docking the ligands were chosen from PubChem, it is a database which is a part of NIH, it contains data about properties, chemical structures and biological activities of small molecules. The selected ligands include Sorafenib, Lenvatinib, Regorafenib, Cabozantinib and Doxorubicin.

2.6 Ligand-Protein Docking

The docking process was carried out using a web-based docking platform called Hdock which produces high performance results. Pymol a widely used visualization software, this software allows visualization of complex structure in 3D it also allows to analyze the interaction between protein and ligand, it provides high resolution images. The docking results were downloaded in PDB file and uploaded in Pymol for visualization.

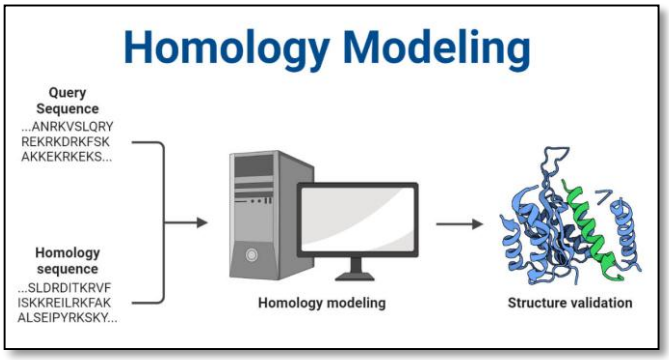


Figure 2.1 Methodology for in-Silico Studies

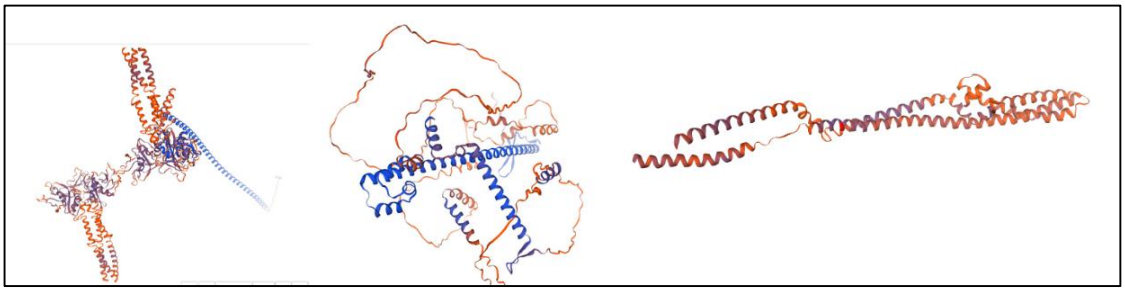
3. Results and Discussion

3.1 Homology Modelling

Selection of target protein was done through NCBI, the sequence was retrieved it FASTA format, the accession codes of the target proteins are **AAI43310.1, AAB32188.1 and AAF76204.2** for CLEC4G, NRF2 and TD26 respectively, the saved FASTA sequence of the template protein and target protein were put in the Clustal omega software to obtain multiple sequence alignment and phylogenetic tree.

3.2 Model Generation

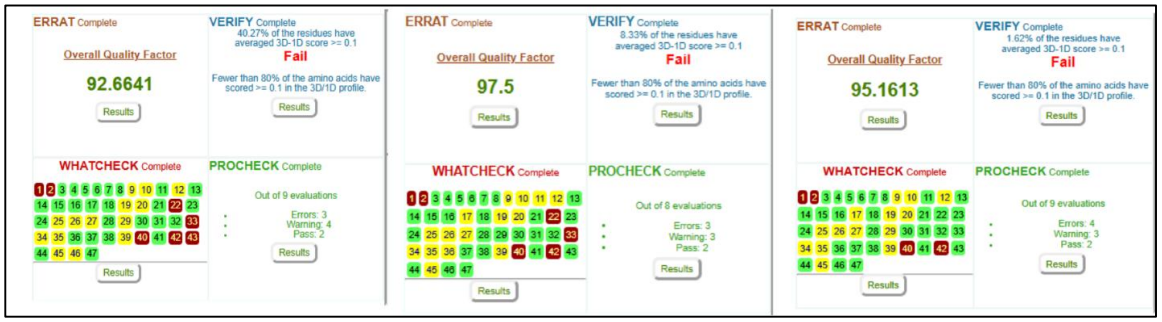
FASTA sequence of the target protein was pasted in the SWISS-MODEL, a tool used for generating homology models of the proteins, it relies of for generating the protein models, the models generated are shown in figure 2.1.



(a) (b) (c)

Figure 3.1 (a) (b) (c) Show the 3D Homology Models of CLEC4G, NRF2 and TD26 Obtained from SWISS-MODEL

The models, were validated in Saves v6.0 software where parameters like ERRAT, VERIFY3D, WHATCHECK and PROCHECK were taken into consideration.

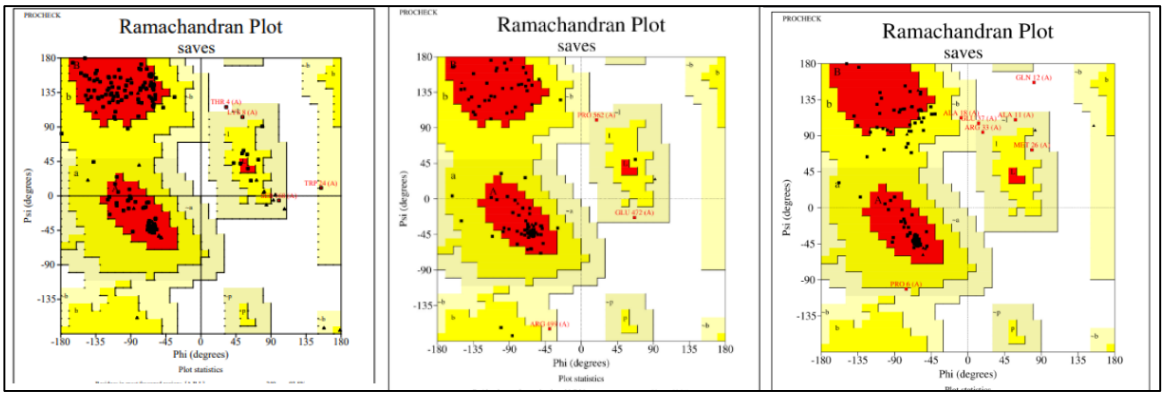


(a) (b) (c)

Figure 3.2 (a), (b), (c) Show the Results Obtained from Saves v6.0

92.6%, 97.5 %and 95.1% ERRAT values as shown in figure 2.2 indicate that the quality of the protein is good. Verify 3D is a technique employed to assess the quality of a protein model by comparing the 3D structure of the protein with its 1D sequence. The protein model is considered to be of high quality if it has a score ≥ 0.2 for more than 80% of its amino acids in the 3D/1D profile, meaning that the amino acid residues are highly supported by the 3D structure.

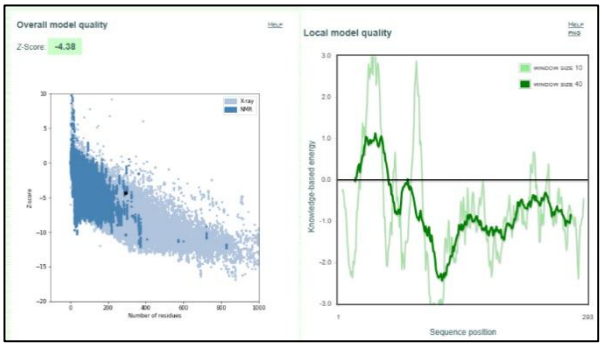
Ramchandran plot is a graphical representation that is used to assess the quality of the generated model. Presence of most of the amino acids in the red region or the favored region shows that the protein is well validated. The result obtained is shown in figure 2.3.



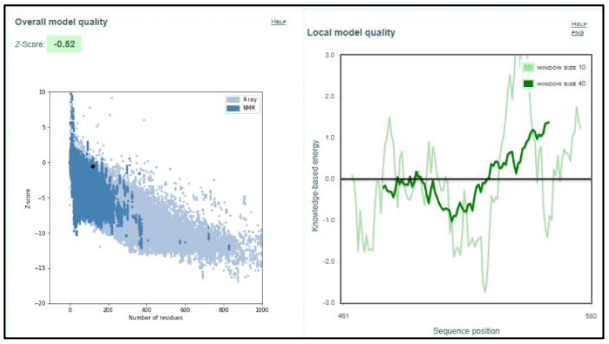
(a) (b) (c)

Figure 3.3 (a) (b) (c): Ramchandran Plot of CLEC4G, NRF2 and TD26 Obtained from saves v60

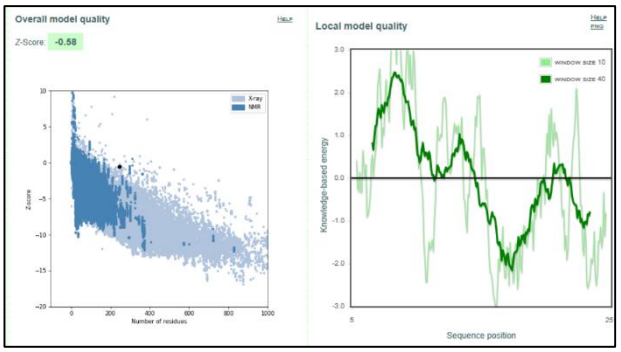
ProSa was used for further validation of the protein model in which the overall and the local was determined. Prosa results are represented in Figure 2.4.



(a)



(b)



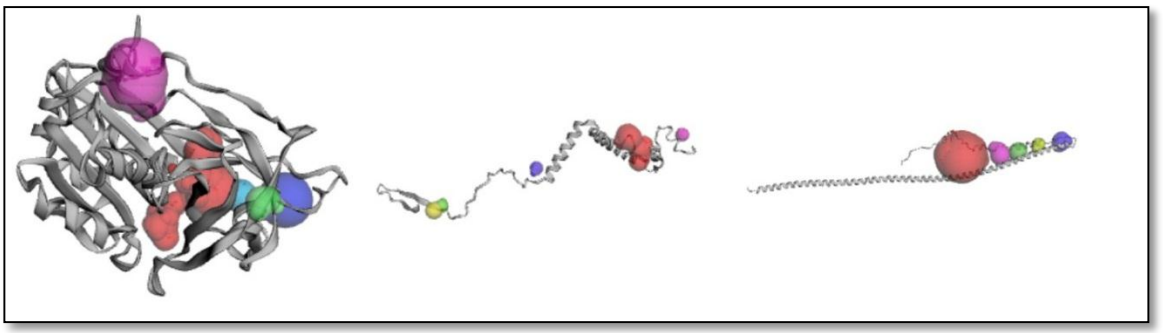
(c)

Figure 3.4 (a) (b) (c) Show the ProSA Results

The ProSa graph shows the energy profile of the protein which is measured as Z-score, the PDB file of the target protein was uploaded to ProSa to obtain the graphs, the negative Z score in the 'overall quality graph' shows that the protein is within the normal energy range, the light blue region indicates X ray crystallography and the dark blue region represents NMR spectroscopy. The 'local quality graph' shows the stability and accuracy of the protein, it represents the energy of the residues.

3.3 Active Site Determination

The active sites of the proteins were obtained using CASTpfold a web-based platform used to determine the active pockets and topological factors of the protein. The protein file was uploaded in PDB file and the active sites were obtained and shown in Figure 2.5.



(a) (b) (c)

Figure 3.5 (a) (b) (c) Represent the Active Pockets of CLEC4G, NRF2 and TD26

3.4 Selection of Ligands

Sorafenib, Lenvatinib, Regorafenib, Cabozantinib and Doxorubicin are drugs used in treatment of HCC, the smile format of these proteins were copied from PubChem and were pasted in Molview for a 3D representation, the structure was downloaded in Mol file and converted into PDB file using open Babel.

3.5 Docking of ligand and macromolecule

Docking of ligands and macromolecule (protein) was performed using Hdock where we uploaded the PDB format of both ligand and protein molecule. The Hdock scores were saved and the file was downloaded and visualized using Pymol. Figures 2.6, 2.7,2.8, 2.9, 2.10, 2.11 show the visual representations of the docking results from Pymol.

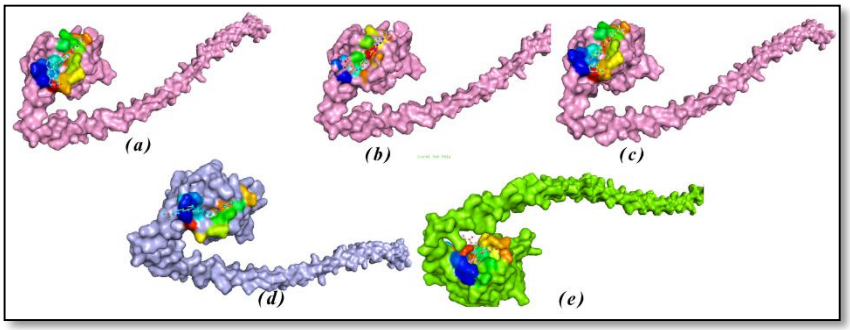


Figure 3.6 (a) (b) (c) (d) (e) docking result of CLEC4G in Pymol, protein is in surface form

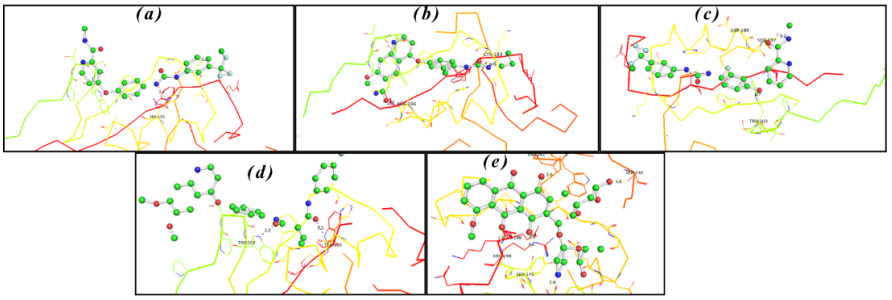


Figure 3.7(a) (b) (c) (d) (e) Show the Visualization of Docking Results between CLEC4G with Sorafenib, Lenvatinib, Regorafenib, Cabozatinib and Doxorubicin from PyMOL Respectively

Table 1 shows the docking score of selected ligands with CLEC4G

CLEC4G	*Sorafenib*	*Lenvatinib*	*Regorafenib*	*Cabozantinib*	*Doxorubicin*
Docking score	***-127.98***	***-131.41***	***-131.42***	***-139.42***	***-153.87***

Based on the table, binding scores of CLEC4G with the five ligands range from –127.98 for sorafenib to –153.87 for Doxorubicin, indicating that CLEC4G shows a strong binding with Doxorubicin.

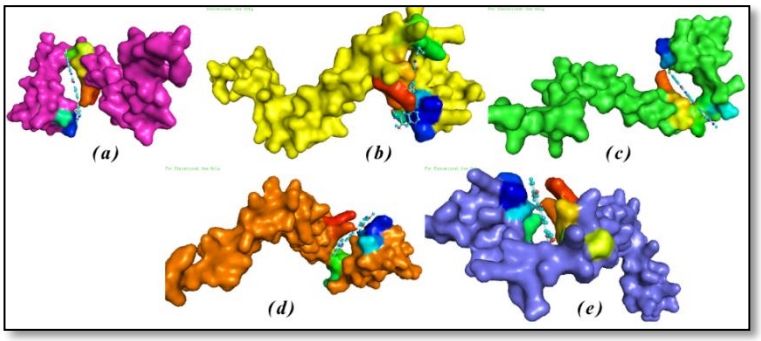


Figure 3.8 (a) (b) (c) (d) (e) Docking Result of NRF2 in Pymol, Protein is in Surface form

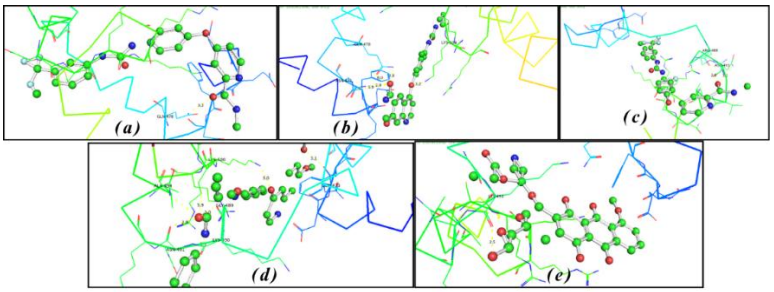


Figure 3.9 (a) (b) (c) (d) (e) Show the Visualization of Docking Results between NRF2 with Sorafenib, Lenvatinib, Regorafenib, Cabozatinib and Doxorubicin from Pymol respectively

Table 2 Shows the Docking Score of Selected Ligands with NRF2

NRF2	Sorafenib	Lenvatinib	Regorafenib	Cabozantinib	Doxorubicin
Docking score	-113.38	-111.21	-116.71	-135.34	-137.42

Table 2 shows the docking scores of NRF2 with ligands, the results tell us that the docking range is between –113.38 for Sorafenib and –137.42 for Doxorubicin, suggesting that Doxorubicin has the highest binding affinity with NRF2 compared to other anti HCC agents.

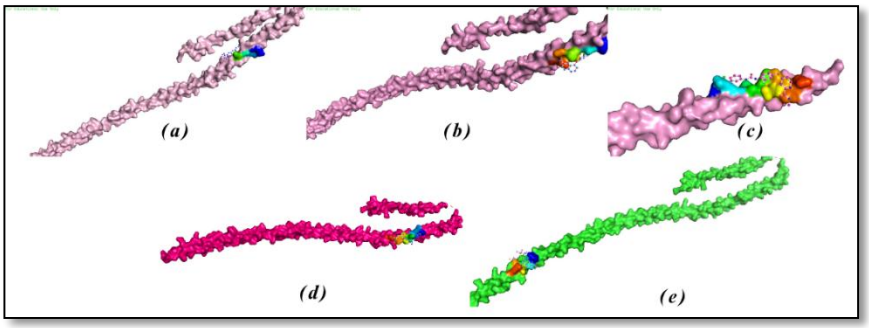


Figure 3.10 (a) (b) (c) (d) (e) Docking Result of TD26 in Pymol, Protein is in Surface form

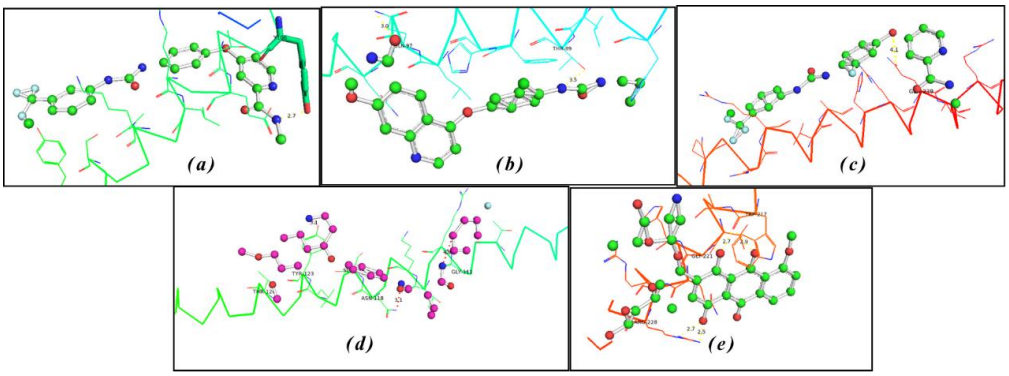


Figure 3.11 (a) (b) (c) (d) (e) Show the Visualization of Docking Results between TD26 with Sorafenib, Lenvatinib, Regorafenib, Cabozatinib and Doxorubicin from Pymol Respect

Table 3 Shows the Docking Score of Selected Ligands with TD26

TD26	Sorafenib	Lenvatinib	Regorafenib	Cabozantinib	Doxorubicin
Docking score	-106.22	-104.07	-104.12	-121.95	-122.77

Based on table 3 we can tell that TD26 shows moderate binding affinity with a range of 106.22 for Sorafenib to –122.77 for Doxorubicin, wherein Doxorubicin showed the strongest binding affinity compared to the rest.

Conclusion

This project provides valuable insight into the docking interaction between CLEC4G, NRF2 and TD26 with anti-HCC agents, showing the potential of these ligands as therapeutic targets, through this study we understood that all the ligands showed promising interactions, wherein doxorubicin has the strongest binding affinity with all the proteins suggesting great protein-ligand interactions. Further lab experiments and clinical trials are needed to completely confirm the therapeutic effect. This research paves a way for designing targeted therapies that may improve the efficacy of cancer treatment.

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