Molecular Docking Studies of 2,4-Dinitrophenylhydrazine from *Thymoquinone* with Liver Cancer Proteins

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Abstract
As one of the main causes of death, liver cancer is a serious threat to global health. Alternative therapeutic options are being investigated as a result of the rising incidence rates of liver cancer and the ongoing toxicity issues with traditional chemotherapeutic medications. In this context, creating new medications from natural sources has become a viable option. The main type of liver cancer, hepatocellular carcinoma (HCC), is closely related to abnormal signaling pathways in the hepatic cellular environment. The development of potent anti-cancer drugs now focuses on addressing these signaling pathways. In order to achieve this goal, the present work used a targeted strategy, docking a thymoquinone derivative, 2,4-dinitrophenylhydrazine, against six key proteins implicated in the evolution of HCC via a grid box with particular dimensions (40Å x 40Å y 40Å z and spacing). SRC, ESR1, TNF and PIK3CA, AKT2, P21 were among the proteins that underwent molecular docking. The thymoquinone derivative showed notable interactions with the chosen proteins in the docking simulations which produced interesting results.

Each protein's minimal binding energies were as follows: PIK3CA (-6.3), TNF (-5.1), ESR1 (-6.1) and SRC (-6.4) AKT2 (-5.0), P21 (-5.1). The corresponding binding distances were also recorded. The notably result was the thymoquinone derivative's strong binding affinity for SRC, PIK3CA and ESR1. These interactions point to the derivative's potential for modifying important signaling pathways linked to HCC.

Keywords: Liver Cancer, Thymoquinone Derivative, 2, 4-Dinitrophenylhydrazine, Molecular Docking.

Introduction

One of the leading causes of death worldwide is cancer which is a powerful enemy of global health[1]. The disturbance of regular cell growth regulatory processes accounts for its intricacy[7]. Liver cancer is one of the most common and deadly types of cancer and it has a major impact on world health. One of the primary treatment approaches has been conventional chemotherapy[8,30]. The severe death rates linked to liver cancer underscore the urgent need for novel therapeutic strategies. In this regard, investigating new agents derived from natural sources, has become more popular as a substitute tactic to improve treatment effectiveness and lessen the problems caused by traditional chemotherapeutic agents.

2, 4-dinitrophenyl hydrazine (DNPH) has been a reliable reagent for identifying carbonyl compounds for almost a century. These molecules can be aliphatic, aromatic, or even proteins and carbohydrates. DNPH solution in 2N hydrochloric acid is the preferred qualitative test for aldehydes and ketones due to its reactivity and selectivity towards the carbonyl group as well as the striking hue of the hydrazones it creates[2]. Exploring the molecular world, single crystal analysis has been used to examine the supramolecular structure of DNPH, revealing details about its complex architecture. Furthermore, DNPH's vibrational spectra have been well investigated in both theoretical and practical investigations, which have helped to characterize it as a well-understood chemical[4,33].

Turning to cellular pathways, the Akt pathway is revealed as an essential signal transduction cascade that coordinates growth and survival in response to external cues[14]. By phosphorylating a variety of intracellular proteins, activated Akt then drives downstream processes that include cell survival, growth, proliferation, migration and angiogenesis[3]. Nevertheless, it is possible for the genes controlling 2, 4-dinitrophenylhydrazine's protective processes to be disrupted, which would prevent cells from going through programmed cell death when it is required.

When combined with additional mutations, this inability plays a crucial role in the development of cancer. This results in dysregulation of the Akt pathway, which prevents the normal planned cell death and permits cancer cells to survive[19,20]. Inflammatory processes and apoptotic developments within cells are significantly influenced by the complex interactions between the Akt pathway and other signaling cascades including NF-κB and Bad. The intricate terrain of cancer development and progression is influenced by the interactions between these pathways[19,20].

Important signaling molecules linked to hepatocellular carcinoma include tyrosine kinase receptors, Ras/Raf/mitogen-activated protein kinase (MAPK), PI3K/Akt/mTOR, Janus kinase (JAK)/Signal transducer and activators of transcription (STAT) and Wnt/β-catenin pathways[5]. Development tailored therapies for liver cancer, with an emphasis on the possible use of 2,4-

dinitrophenylhydrazine, requires an understanding of how various signaling pathways interact.

2, 4-dinitrophenylhydrazine has a variety of anti-cancer actions that affect several pathogenic processes in cancer cells. Notably, it has a significant impact on cell junctions, inflammation and apoptosis[11]. According to Li et al[11], 2, 4-dinitrophenylhydrazine inhibits the growth of hepatocellular carcinoma cells and triggers apoptosis via a signaling pathway that includes the transcriptional activation of Bcl2 and Bax. The complex regulatory significance of 2, 4-dinitrophenylhydrazine in deciding the destiny of cancer cells is demonstrated by this dual modulation of pro-apoptotic and anti-apoptotic proteins. Furthermore, liver cancer treatments utilizing 2,4-dinitrophenylhydrazine show interest in targeting transcription factors including nuclear factor-kappa B (NF-κB) and cell cycle regulators like cyclins and cyclin-dependent kinases (CDKs)[5].

The capacity 2,4-dinitrophenylhydrazine's capacity to affect these important molecular actors points to its potential as a versatile therapeutic agent that can interfere with the intricate web of signaling pathways and regulatory components linked to the development of liver cancer. The foundation for future studies aimed at creating focused tactics to fight hepatocellular carcinoma is laid by the thorough comprehension of these molecular interactions.

Material and Methods

Experimental Method: A new 2, 4-dinitrophenyl hydrazine from thymoquinone was evaluated for its ability to cause liver cancer via molecular docking experiments.

Preparation of Ligand: The PubChem database provided the 2,4-dinitrophenylhydrazine's chemical structure in sdf format. The sdf format was then converted into the.PDB format using an online SMILES converter. Following that, ligand structures were examined using AutoDockTools (ADT), with particular attention paid to their interactions with rotatable bond structures, Gasteiger changes and non-polar hydrogens. Using ADT's assistance, the PDB files were further converted into the ligand format, more precisely, PDBQT format, to enable interoperability with AutoDock4 (AD4) and AutoDock Vina. For next molecular docking investigations, this format change is essential. The entire procedure followed the guidelines provided by Morris et al[17] guaranteeing a methodical and verified approach to the ligands' structural characterization.

Preparation of Protein: From the RCSB Protein Data Bank (http://www.rcsb.org), we obtained the crystal structures of five proteins linked to liver cancer. The atomic coordinates from the downloaded PDB data had to be extracted in order to prepare protein structure inhibitors. We used the AutoDockTools (ADT) program to expedite the AutoDock Vina preparation. ADT was used to improve the protein structures in a number of important processes. In order to concentrate on the interactions between the protein and the ligand, it was necessary to remove the water molecules.

Additionally, ADT performed the conversion of protein structures from the .PDB file format to the PDBQT file format, computed Gasteiger charges on these structures and assisted in assigning hydrogen polarities to protein structures. A crucial pre-requisite for further molecular docking research is compatibility with AutoDock Vina, which is ensured by this format change. This structural preparation's methodology followed Villanuevain et al's[34] recommendations, guaranteeing a verified and consistent procedure for optimizing protein structures for docking research. We hope to improve the precision and dependability of ensuing analyses by meticulously selecting and honing the crystal structures of the chosen proteins, thereby fostering a solid comprehension of the molecular connections in pathways linked to liver cancer.

Preparation of Grid Box: According to Sharma et al[27,28] and Ratra et al[23] determining the ideal grid arrangement is an essential step in docking investigations.

In order to cover the complete binding pocket of the target protein, we chose the best grid structures and coordinates. This deliberate choice guarantees thorough coverage of all possible contact sites which are essential for precise molecular docking. The grid parameter file that resulted from loading the prot.pdbqt file into the grid macromolecule in AutoDock was saved as a configuration (conf) file. Later docking studies relied heavily on this conf file, which streamlined the procedure for reproducibility and consistency. A key component of the study, the computed grid dimensions, are shown in table 1 to provide light on the spatial parameters used in the docking simulations.

Table 1
Dimensions of Grid Box of 2, 4-Dinitrophenylhydrazine's Compounds

Proteins Name		**SRC**	**ESR1**	**TNF**	**PIK3CA**	**AKT2**	**P21**
Grid Dimension	Size x	40	40	40	40	40	40
	Size y	40	40	40	40	40	40
	Size z	40	40	40	40	40	40
	Center x	12.544	7.356	7.975	36.218	99.962	30.976
	Center y	3.176	12.582	34.604	0.7022	22.035	99.268
	Center z	18.648	12.995	16.293	11.110	38.7	25.955

To improve the accuracy and dependability of our docking studies, we sought to optimize the grid configuration by following accepted practices and utilizing AutoDock Tools. A more detailed knowledge of the molecular interactions underlying the targeted protein-ligand interactions is eventually made possible by this careful grid arrangement, which guarantees a thorough investigation of the binding pocket.

Each of the necessary files, namely, lig.pdb, lig.pdbqt, prot.pdb, prot.pdbqt and conf.txt, was painstakingly assembled and saved. The molecular docking results were then acquired, producing a log file in addition to the ligand pdbqt files. PyMOL software was used to display the location of the ligand with the highest negative binding energy, which was stored as a complex file in pdb format. The saved complex file was subjected to additional analysis in Discovery Studio to better explore the complex interactions between the ligand and protein. The investigation of molecular interactions in two-dimensional (2D) and three-dimensional (3D) diagrams was made easier facilitated by this platform. In addition to guaranteeing thorough documentation of the docking results, this method enables visualization of the binding dynamics between the ligand and the target protein in great detail.

Molecular Docking Study: The AutoDock Vina application was used in the docking process to dock molecules. Different grid box sizes and grid coordinates (grid center) were used for ligand docking for each receptor. The ligand remained in a flexible state when it interacted with macromolecules in rigid environments. Notepad was used to access the configuration file in order to run Auto Dock Vina. AutoDockTools (ADT) is essential for setting up the size and center of the grid box and generating the input PDBQT file for proteins. Polar hydrogen atoms and Kollman charges are present in protein structures. Docking was carried out with the grid box's default settings and PDBQT was used to save the produced file.

AutoDock Vina predicted ligand binding affinities and provided negative Gibbs free energy (ΔG) scores in kcal/mol. PyMOL was used for postdocking analysis, which clarified the sizes, locations and hydrogen-bond interactions of the binding sites. Compounds docked at the active areas of target proteins. Each ligand's binding position was then carefully examined to determine how it interacted with the protein. Each ligand's optimal and most energetically advantageous conformations were carefully chosen. In addition to the use of advanced computational techniques for precise docking, this methodical approach enables a thorough postdocking study that clarifies the interactions and dynamics of ligand-target protein binding.

Preparation of SRC, ESR1, TNF and PIK3CA, AKT2, and P21 structures: SRC, ESR1, TNF and PIK3CA, AKT2, and P21 structures were generated via information from the Protein Data Bank [PDB:4K11, 1UOM, 6OOY, 6PYS, 2AM9 IMVH1, 2COW] (http://www.rcsb.org). The atomic coordinates of the protein structure inhibitors were extracted from the pdb file and any water molecules including those that cocrystallized with the protein, were then eliminated. AutoDockTools (ADT) was used to prepare these protein structures for AutoDock Vina. Assigning hydrogen polarity, computing Gasteiger charges for the protein structure and transferring the protein structure from pdb file format to pdbqt format were all essential phases in this process, which was carried out in accordance with established standards.

The implementation of the methods described by Morris et al[17], Lim et al[12] and Jaghoori et al[10] guaranteed a thorough and uniform method for preparing the protein structures for subsequent molecular docking investigations. To maximize the precision and dependability of the docking simulations and lay the groundwork for a comprehensive analysis of the interactions between the chosen proteins and possible ligands, this pain staking preparation procedure is essential.

Results and Discussion

SRC, ESR1, TNF, PIK3CA, AR, AKT2, and P21 are important proteins implicated in apoptosis. The test material, 2, 4-dinitrophenylhydrazine and positive control drugs were subjected to molecular docking analysis. The effective docking of apoptotic proteins with 2,4-dinitrophenylhydrazine was confirmed by calculating the minimal binding energy. SRC (-6.4), ESR1 (-6.1), TNF (-5.1), PIK3CA (-6.3), AKT2 (-5.0), and P21 (-5.1) were among the binding energies of 2, 4-dinitrophenylhydrazine that showed remarkable affinities (Table 2). In contrast, the drugs that tested positive, had the lowest binding energy. These convincing results highlight the significant binding affinity of 2,4-dinitrophenylhydrazine and its positive interactions with apoptotic proteins.

Table 2
Binding Energies (B.E) Between the Proteins and Ligand

S.N.	Target	PDB ID	Binding Energy Kcal/Mol (BE)
1.	SRC	4K11	-6.4
2.	ESR1	IUOM	-6.1
3.	TNF	6OOY	-5.1
4.	PIK3CA	6PYS	-6.3
5.	AKT2	IMVH1	-5.0
6.	P21	2COW	-5.1

Interestingly, the computed binding energies of 2,4-dinitrophenylhydrazine are greater than those of positive control drugs, indicating greater stability inside the liver cancer protein cavity. This observation suggests that 2,4-dinitrophenylhydrazine has improved anticancer properties.

Generation of Output and Visualization of Docked Complexes: The results of 2,4-dinitrophenylhydrazine's molecular docking with the liver cancer-related proteins SRC, ESR1, TNF and PIK3CA, AKT2 and P21 were carefully investigated. This thorough investigation clarifies the complex molecular connections found in the liver cancer proteins, offering important new information about the possible therapeutic benefits of 2, 4-dinitrophenylhydrazine.

Apoptotic Proteins with PDB ID and Binding Energy: Promising information about the possible therapeutic impact of 2, 4-dinitrophenylhydrazine was revealed by the molecular docking analysis of the compound within the binding site of EGFR which is situated in the binding pocket of the receptor protein. Notably, the SRC protein is essential for fostering cell growth, proliferation and motility and is often activated in liver cancer[16,22,29]. The importance of SRC expression in the development of liver cancer is further highlighted by the fact that its dysregulation is linked to cancer cell resistance.

TNF-α-induced IKB kinase activation is suppressed and a direct contact with the p65 subunit is involved in the inhibitory effect on NF-κB. Anti-apoptotic and proliferative regulatory proteins are also affected by this complex-mediated inhibition of NF-κB activation which is triggered by a variety of carcinogens and inflammatory stimuli[26].

According to molecular docking studies, 2,4-dinitrophenylhydrazine may be able to successfully inhibit the expression of the SRC protein as well as the activation of the TNF protein in liver cancer cells. Additionally, docking experiments indicate that the protein SRC might have more potent inhibitory effects with 2, 4-dinitrophenylhydrazine than the TNF protein does. These results pave the way for further research on the application of these substances in liver cancer cells. This study provides thorough molecular insights into the potential of ,4-dinitrophenylhydrazine as a therapeutic agent for the treatment of liver cancer and provides a more nuanced understanding of how it interacts with important proteins.

By focusing on the PIK3C and ESR1 pathways, this ground-breaking docking study highlights the critical function that 2, 4-dinitrophenylhydrazine plays in significantly reducing the growth of liver cancer cells. Mutations that activate the p110α subunit of PI3K (PIK3CA) have been found in a wide range of tumor types. According to previous analyses, PIK3CA mutations enhance the PI3K signal, promote growth factor-independent growth, activate downstream Akt signaling and increase cell invasion and metastasis. Drug-resistant cancer cells exhibit substantial overexpression of the ESR1 protein, which complicates the treatment of cancer. The increased expression of ESR1 acts as a protective mechanism against drug-mediated apoptosis, even while it contributes to the cleavage of transcripts that reduce cell survival.

ESR1 plays a crucial role in the development of cancer and is implicated in liver cancer because it affects proliferation, angiogenesis and tumor vascular density. When the ESR1 gene is activated, the ER protein may be overactive or more abundant than usual in cancer cells. As a result, cancer cells may proliferate, spread and become resistant to some anticancer medications.

By performing a docking research, this work expands on earlier mechanistic discoveries in the context of liver cancer and offers concrete evidence the potential of 2, 4-dinitrophenylhydrazine. The main goal is to bind to the protein's binding site, which controls apoptosis. The docking results, taking into account binding scores and inhibitory activity against ESR1 and PIK3CA, provide important evidence for the possible mechanisms of action of 2, 4-dinitrophenylhydrazine in liver cancer. Analysis revealed that PIK3CA exhibits has good activity against drug-resistant processes and has a greater binding affinity than ESR1 does.

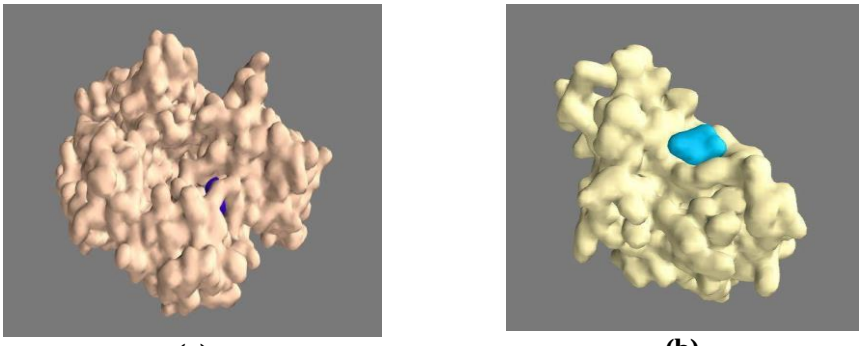
The current binding sites provide more evidence in favour of these chemicals' possible use in the treatment of liver cancer. The recorded outcomes, as shown in table 3 and figure 2, provide a basis for further research and call for a more thorough analysis of these substances' actions in liver cancer cells.

Table 3
Binding Energy between 2, 4-Dinitrophenylhydrazine with SRC and TNF Proteins

S.N.	Target	PDB ID	Binding Energy Kcal/Mol (BE)
1.	SRC	4K11	-6.4
2.	TNF	6OOY	-5.1

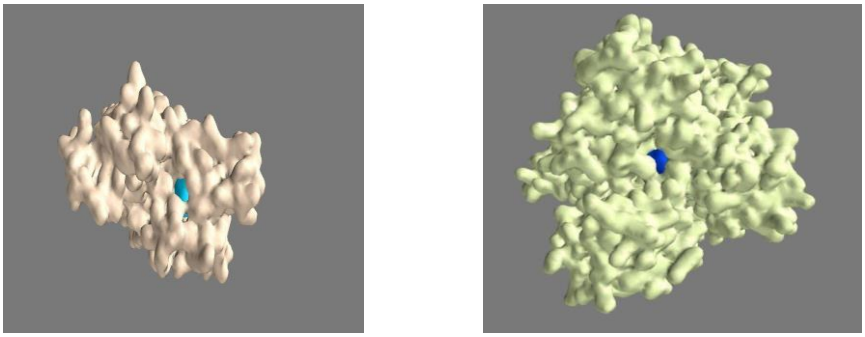
Table 4
Binding Energy between 2, 4-Dinitrophenylhydrazine with ESR1 and PIK3CA Proteins

S.N.	Target	PDB ID	Binding Energy Kcal/Mol (BE)
1.	ESR1	IUOM	-6.1
2.	PIK3CA	6PYS	-6.3



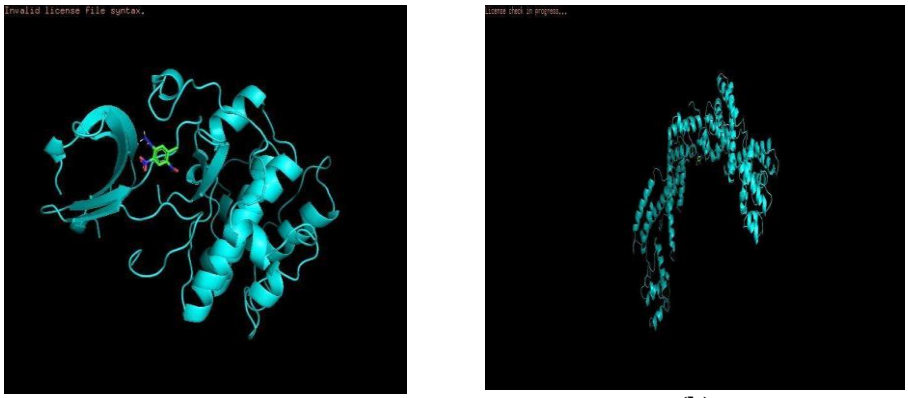
(a) **(b)**

Figure 1: (a) and (b) Docking pose of 2,4-Dinitrophenylhydrazine with SRC and



(a) **(b)**

Figure 2: (a) and (b) Docking of the 2, 4-Dinitrophenylhydrazine with ESR1 and PIK3CA



(a) **(b)**

Figure 3: (a) and (b) Docking of the Ligands, 2, 4-Dinitrophenylhydrazine with AKT2 and P21 into the Binding Pocket of Survivin

Table 5
Binding Energy between 2, 4-Dinitrophenylhydrazine with AKT2 and P21 Proteins

S.N.	Target	PDB ID	Binding Energy Kcal/Mol (BE)
1.	AKT2	IMVH1	-5.0
2.	P21	2COW	-5.1

The onset and spread of liver cancer, especially hepatocellular carcinoma (HCC), have been linked to Akt2. Akt2 activates downstream pathways to support cell survival and proliferation. Akt2 stimulates invasion and migration of cells, both of which are essential for the spread of cancer. Angiogenesis, which is necessary for tumor growth and metastasis, is regulated by Akt2. P21 has both tumor-promoting and tumor-suppressive properties which contribute to its complex role in liver cancer. P21 suppresses the advancement of the cell cycle which can stop the growth of cancer cells and can ultimately eradicate them. P21 has the ability to cause cancer cells to undergo apoptosis which can eradicate harmful cells. Using a computational method, in silico molecular docking to 2, 4-dinitrophenylhydrazine apoptotic pathways such Akt2 and P21 was carried out to illustrate how thymoquinone functions as an anticancer medication. We calculated the binding distance and minimal energy.

Conclusion

To carefully determine the interaction between ligands and proteins linked to liver cancer, the current study range of molecular docking binding energies. To anticipate the effectiveness of possible treatment drugs, binding energies must be evaluated; larger negative values signify stronger binding to the target location. Throughout our study, 2, 4-dinitrophenylhydrazine continuously demonstrated strong affinity for the target proteins with outstanding negative binding energies between -6.4 and -5.0 kcal/mol. Our results are consistent with those of other studies, supporting the idea that 2, 4-dinitrophenylhydrazine has an excellent binding capability confirming its possible use in liver cancer research.

The thorough investigation of its binding relationships with important receptor and apoptotic proteins, such as SRC, ESR1, TNF and PIK3CA, AKT2 and P21 highlights the adaptability of 2, 4-dinitrophenylhydrazine and its derivatives. In the field of liver cancer research and development, 2, 4-dinitrophenylhydrazine is positioned as a prospective lead chemical because of its demonstrated effectiveness against a variety of apoptotic and receptor proteins. The study's molecular insights provide a basis for future research, promoting the analysis of synthetic chemicals produced from 2, 4-dinitrophenylhydrazine against a range of molecular targets implicated in the pathways leading to liver cancer.

By focusing on receptors essential to the development of cancer and important proteins linked to apoptosis, 2, 4-dinitrophenylhydrazine becomes a flexible option for therapeutic intervention. We deduce that both SRC and PIK3CA exhibit potent ligand binding activity which influences drug resistance and cell proliferation.

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