

Exploring the Molecular Mechanisms of Senegenin in Lung Cancer via PI3K/AKT/mTOR Pathway and Computational Docking Studies

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ABSTRACT

Background: Lung cancer is a pervasive global malignancy that stands as a foremost cause of cancer-induced fatalities, defined by its heightened intratumor heterogeneity and the intricate nature of its cellular pathology. It accounts for an estimated 25% of all global cancer-related fatalities, making it the primary cause of cancer deaths despite the development of clinical treatments. **Objectives:** The present work was designed to evaluate the anticancer mechanisms of senegenin against lung cancer A549 cells using an *in vitro* and *in silico* approach. **Materials and Methods:** The MTT test was utilized to examine the growth of lung cancer A549 cells treated with senegenin at different concentrations (5-30 μ M). The incidences of apoptotic cell deaths in the senegenin-treated A549 cells were evaluated using a dual staining method. The levels of PI3K, Akt, and mTOR in the control and senegenin-treated A549 cells were evaluated with appropriate assay kits. The interactions between the senegenin and the PI3K, Akt, and mTOR were evaluated using Autodock vina and visualized using Discovery Studio Visualizer (DSV). The drug-likeness and toxicity profile of senegenin were predicted using ProTox v3.0. **Results:** The findings of the MTT assay indicated that senegenin treatment (5-30 μ M) significantly reduced the A549 cell viability in a dose-dependent manner. The dual staining results indicated the increased presence of apoptotic incidences in the senegenin-treated A549 cells. The levels of PI3K, Akt, and mTOR were markedly decreased in the A549 cells after treatment with senegenin. The molecular docking analysis revealed that senegenin exhibited strong interactions with the key components of the PI3K/Akt/mTOR signaling pathway such as PI3K (Binding Affinity (BA) = -7.4 kcal/mol; RMSD = 2.263Å), AKT (BA = -9.0 kcal/mol; RMSD = 2.493Å), and mTOR (BA = -7.4 kcal/mol; RMSD = 2.613Å). **Conclusion:** The current findings suggest that senegenin interacts with PI3K, Akt, and mTOR and may potentially inhibit downstream signaling events associated with cell growth, proliferation, and survival in lung cancer progression. The docking results supported the hypothesis that senegenin could act as a multi-target inhibitor to modulate the oncogenic PI3K/Akt/mTOR signaling pathway. Consequently, it may be a promising anticancer candidate for treating lung cancer.

Keywords: A549 cell line, Apoptosis, Lung cancer, PI3K/Akt/mTOR pathway, Senegenin.

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INTRODUCTION

Lung cancer, a widespread global malignancy, continues to be a primary contributor to cancer-related mortalities, largely due to its significant intratumor heterogeneity and the complex cellular characteristics inherent to the disease.¹ Lung cancer is characterized by uninhibited cell proliferation in lung tissues and have the capacity to metastasize to distant organs if left untreated. It accounts for an estimated 25% of all global cancer-related

fatalities, making it the primary cause of cancer deaths despite developments in early diagnosis and clinical treatments. This places lung cancer as the most widely diagnosed cancer and the major of cancer-associated deaths worldwide.² The global incidence and death rates of lung cancer are predicted to increase substantially, with estimates reaching 4.62 million new cases and 3.55 million mortalities by 2050 if current trends persist. The prevalence of lung cancer is disproportionately high in Asia and among male individuals, with cigarette smoking remaining the primary etiological factor globally. However, other contributing factors include exposure to harmful airborne particles, genetic predispositions, and advancing age. Although other risk factors contribute to lung cancer, smoking is the primary cause, responsible for more than 85% of cases worldwide.³ Despite the declining incidence rates in some developed nations, which align with a reduction in smoking prevalence, lung cancer remains the



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primary cause of cancer deaths globally. This persistent burden necessitates comprehensive strategies for prevention, early detection, and advanced therapeutic interventions tailored to specific regional and demographic contexts.⁴

The uncontrolled cellular proliferation characteristic of malignant tumors often involves the evasion of apoptosis, a critical mechanism in maintaining tissue homeostasis and preventing carcinogenesis.⁵ The dysregulation of apoptotic pathways enables cancerous cells to bypass normal cellular checkpoints, resulting in sustained survival and unchecked replication. A significant mechanism by which lung cancer cells achieve this evasion is through the overexpression of anti-apoptotic proteins, rendering them less susceptible to therapeutic interventions designed to induce cell death.⁶ The PI3K/Akt/mTOR pathway, a key regulator of cell survival, proliferation, and metabolism, is frequently dysregulated in lung cancer, contributing to apoptosis evasion and therapeutic resistance. In normal cells, this pathway maintains homeostasis, but in cancer cells, its hyperactivation promotes survival and proliferation. Akt, a central component, phosphorylates and inhibits pro-apoptotic proteins like BAD and caspase-9, while mTOR enhances survival signaling and protein synthesis, contributing to chemotherapy resistance.⁴⁵ Notably, the PI3K/Akt/mTOR axis is aberrantly activated in non-small cell lung cancer (NSCLC), the most common subtype, and correlates with poor prognosis and drug resistance. In A549 cells, this pathway drives growth, survival, and resistance to therapy. Targeting PI3K/Akt/mTOR offers a promising strategy to overcome apoptosis evasion and enhance treatment efficacy.⁷

Current primary treatments, including surgery, chemotherapy, radiotherapy, and targeted therapy often yield promising results in early-stage lung cancer; however, these conventional approaches are frequently associated with severe side effects and a high risk of recurrence, particularly in advanced stages.⁸ The challenges associated with these conventional treatments, such as systemic toxicities and the onset of drug resistance, necessitate the exploration of novel therapeutic avenues, particularly those derived from natural sources that may offer enhanced efficacy with reduced adverse effects.^{9,10} Senegenin is a primary bioactive constituent found in the roots of *Polygala tenuifolia*, has demonstrated several biological properties. It has already been reported that senegenin attenuated pulmonary fibrosis,¹¹ exerted neuroprotective effects,¹² ameliorated acute lung injury,¹³ inhibited neuronal injury,¹⁴ and exerted anti-depression¹⁵ activities in various experimental models. Furthermore, it has been suggested that senegenin treatment inhibited cell growth and metastasis in hepatocellular cancer.¹⁶ Nevertheless, there are no previous reports on the anticancer activity of senegenin against lung cancer. Consequently, the present work was designed to evaluate the anticancer mechanisms of senegenin against lung cancer A549 cells through the PI3k/Akt/mTOR metabolic pathway axis.

MATERIALS AND METHODS

In vitro

Maintenance of cell culture

The lung cancer A549 cell line was acquired from ATCC, USA, and cultured in DMEM media enriched with 10% FBS and 1% antimycotic mixture in a 5% CO₂ incubator. Cells were harvested upon reaching 80% confluency and employed for subsequent fluorescence staining and biochemical experiments.

MTT assay

The cytotoxic effects of senegenin on the growth of A549 cells were evaluated using MTT assay. The cells were cultivated on a 96-well plate for 24 hr and subsequently cells were subjected to senegenin treatment for 24 hr at a dosage of 5, 10, 15, 20, 25, and 30 μ M, respectively. After treatments, 20 μ L of MTT reagent and 100 μ L of DMEM were introduced to each well for a duration of 4 hr. After dissolving the produced formazan deposits with DMSO (100 μ L), the absorbance was measured at 570 nm.

Dual staining

The control and senegenin-treated A549 cells were analyzed for apoptotic incidences utilizing a dual staining method. A 24-well plate was utilized to culture the cells prior to treatment with 20 μ M of senegenin for 24 hr. Each well was filled with 100 μ g/mL of AO/EB dye combination and incubated in the dark for 5 min to evaluate apoptosis in A549 cells. A fluorescent microscope was employed to assess the generated fluorescence in the cells.

Analysis of PI3K, Akt, and mTOR levels

The control and senegenin-treated A549 cells were acquired and homogenized with cell lysis buffer to prepare the cell lysate. The produced cell solution was centrifuged at 6000 rpm for 10 min, and the resultant supernatant was obtained for the analysis of PI3K/Akt/mTOR pathway protein levels. The concentrations of PI3K, Akt, and mTOR in the cell lysates of both control and senegenin-treated A549 cells were analyzed using the appropriate assay kits, adhering carefully to the manufacturer's instructions (MyBioSource, USA).

In silico

The dataset retrieval and its preparation for molecular docking analysis

The targets such as PI3K (PDB ID: 6ZAD),¹⁷ Akt (PDB ID: 6CCY),¹⁸ and mTOR (PDB ID: 4DRI)¹⁹ were obtained from the Protein Data Bank (PDB) and pre-processed by removing water and associated ligands using Discovery Studio Visualizer (DSV) v19.1.0.18287 (www.accelerys.com) to perform the molecular docking analysis. This software is available at Rue Marcel Dassault, Vélizy-Villacoublay, France (URL: www.accelerys.com, Accessed on 18 Nov 2025). Likely, the senegenin structure was downloaded from the PubChem compound database along with

its 3-dimensional (3D) atomic co-ordinates and then converted into the PDB format using DSV.²⁰

Drug likeness and Toxicity properties prediction

The drug likeness and ADMET properties were predicted for the senegenin using the ProTox 3.0 (<https://tox.charite.de/prottox3/index.php?site=home>; Accessed on 19 Nov 2025).²¹

Molecular docking and Visualization

The molecular docking was performed for the defined targets, PI3K, Akt, and mTOR with senegenin by implementing Autodock Vina, an advanced tool with an integrated scoring algorithm integrated into PyRx software.²² This process predicts the most favorable binding modes between the defined targets and the ligand.

The Autodock vina uses a new scoring mechanism,

$$C = \sum_{i < j} f_{titj}(r_{ij}),$$

C- Sum of intermolecular and intramolecular distance; Σ - Over all of the pairs of atoms; f_{titj} - Symmetric set of interaction functions; r_{ij} - Interatomic distance

The selected targets and ligand have been further converted into PDBQT format and assigned grid box as follows: PI3K (size_x = 114.27Å; size_y = 92.90Å; size_z = 84.09Å), Akt (size_x = 64.43Å;

size_y = 60.02Å; size_z = 71.68Å), and mTOR (size_x = 50.07Å; size_y = 53.33Å; size_z = 43.54Å). After running molecular docking, we analyzed the PI3K, Akt, mTOR interactions with senegenin in 3D and 2D format using DSV and validated with LigPlot+ v2.3.1.

Statistical analysis

Statistical tests were performed using GraphPad Prism software, and the results are expressed as the mean $\pm$ SD of triplicates. One-way ANOVA and Tukey's post hoc assay were conducted to assess the values, with a significance established at $p < 0.05$. Each experiment was performed in triplicate ($n=3$ biological replicates) with each experiment having 3 technical replicates. The calculation for IC₅₀ level of senegenin was done using GraphPad Prism software.

RESULTS

Effect of senegenin on the viability of lung cancer A549 cells

Figure 1 illustrates the cytotoxic effects of senegenin on the viability of A549 cells. Senegenin treatment at several concentrations (5-30 μ M) markedly diminished the vitality of A549 cells at dose-dependently. The IC₅₀ concentration of senegenin was established at 20 μ M, resulting in a 50% inhibition of cell proliferation. Consequently, 20 μ M of senegenin were selected as the IC₅₀ dosage for further investigations.

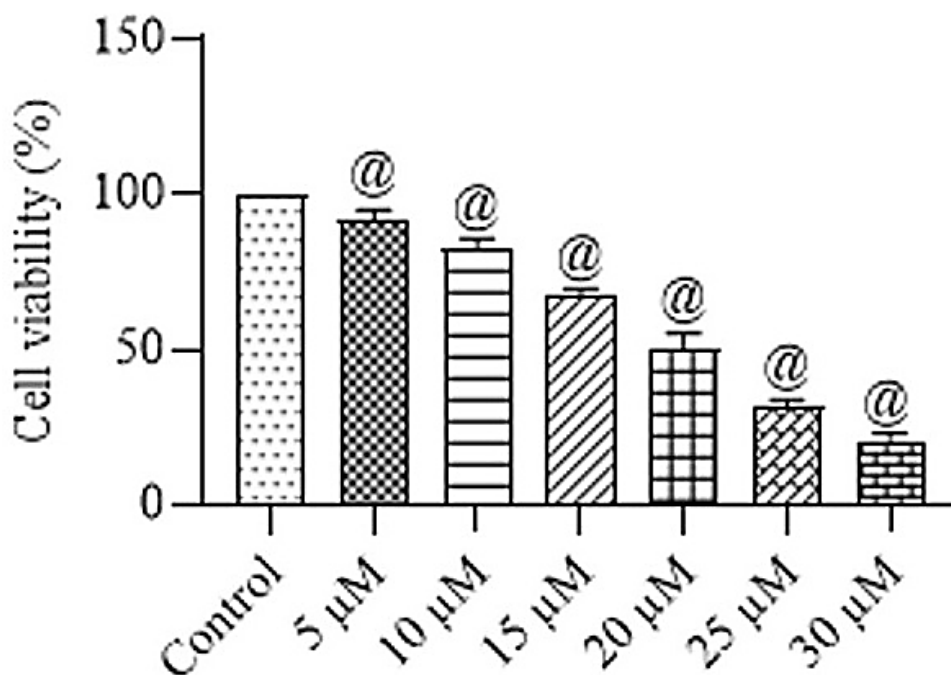


Figure 1: Effect of senegenin on the viability of lung cancer A549 cells. The treatment with various concentrations (5-30 μ M) of senegenin significantly inhibited the A549 cell growth at dose-dependently. The IC₅₀ value of senegenin against A549 cells was determined to be 20 μ M. The findings are presented as Mean $\pm$ SD of triplicate assays, with each value analyzed using one-way ANOVA and Tukey's *post hoc* assay. "@" indicates that the data in treatment groups significantly differ from the control group at $p < 0.05$.

Control

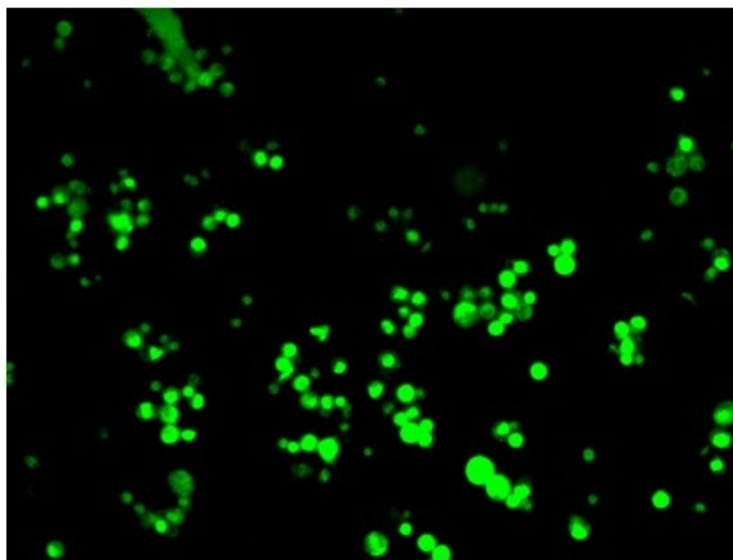
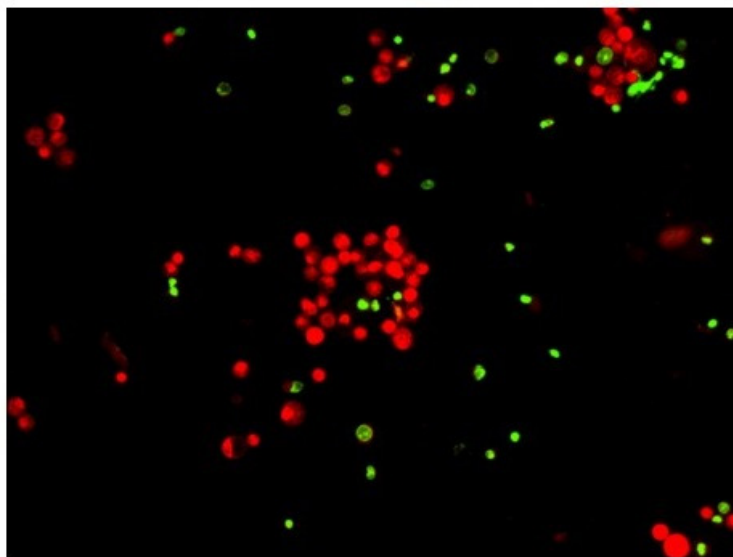
20 μ M

Figure 2: Effect of senegenin on the apoptotic levels in the A549 cells. The control cells displayed intense green fluorescence, indicating the presence of viable cells without apoptosis. Following 24 hr of treatment with 20 μ M of senegenin, a considerable increase in yellow/orange fluorescence was seen, indicating a higher incidence of both early and late stage apoptosis in lung cancer A549 cells.

Effect of senegenin on the apoptotic levels in the A549 cells

The incidence of apoptotic cell deaths in untreated and senegenin-treated A549 cells was assessed by AO/EB staining, with the findings illustrated in Figure 2. Senegenin treatment at 20 μ M dosage in A549 cells resulted in an elevation in apoptotic incidences relative to the untreated control. The intensity of yellow/orange fluorescence in senegenin-treated cells significantly exceeded that of the control cells, indicating that senegenin enhances apoptosis in lung cancer cells.

Effect of senegenin on PI3K/Akt/mTOR protein levels in the A549 cells

Figure 3 illustrates the findings of an analysis of PI3K, Akt, and mTOR protein levels in both control and senegenin-treated A549

cells. Treatment with 20 μ M of senegenin significantly decreased the PI3K, Akt, and mTOR protein levels in the A549 cells when compared with the control. Consequently, it was evident that senegenin treatment could downregulate the protein levels of PI3K/Akt/mTOR in the lung cancer cells.

Molecular docking analysis

The molecular docking analysis performed for the PI3K, Akt, and mTOR showed that senegenin can interact with Akt more well than the PI3K and mTOR. The obtained binding affinity, RMSD, and the types of bonds involved in the interactions of the PI3K, Akt, mTOR with the senegenin were tabulated in Table 1.

PI3K

The senegenin interacted with the residues of PI3K, including THR458, ALA456, and SER990 via the hydrogen bonds and carbon hydrogen bond. Also, the alkyl bond formed with ALA456 with the binding affinity of -7.4 kcal/mol and the RMSD was observed as 2.263Å. Further, the residues such as TRP420, VAL445, ASN454, GLY457, LYS620, TYR621, VAL657, ARG982, ALA983, GLY985, SER990, and CYS991 stabilizes the complex by interacting hydrophobically with the senegenin (Figures 4A, 4B and Table 1).

Akt

Akt residue such as LYS158, GLU198, and GLU191 interacted with the senegenin with the binding affinity of -9.0 kcal/mol and the RMSD was observed as 2.493Å. Meanwhile, the

residues PHE161 and VAL164 interacted via the alkyl, pi-alkyl, and pi-sigma bonds. Also, the hydrophobic residues such as GLY157, GLY159, GLY162, LYS163, LYS179, LEU181, ILE186, HIS194, GLU234, GLU278, ASN279, ASP292, GLY294, and LEU295 surrounded the complex and additionally stabilizes the senegenin-Akt interactions (Figures 4C, 4D and Table 1).

mTOR

The senegenin interacted with residues such as ASP2102 via the hydrogen bond with the binding affinity of -7.4 kcal/mol and the RMSD was observed as 2.613Å. Also, the other residues such as PHE2039, TRP2101, and TYR2105 interacted with the senegenin via pi-sigma and pi-alkyl bonds. Additionally, the residues such as TYR2038, VAL2094, and THR2098 surrounded the complex via hydrophobic interactions (Figures 4E, 4F and Table 1).

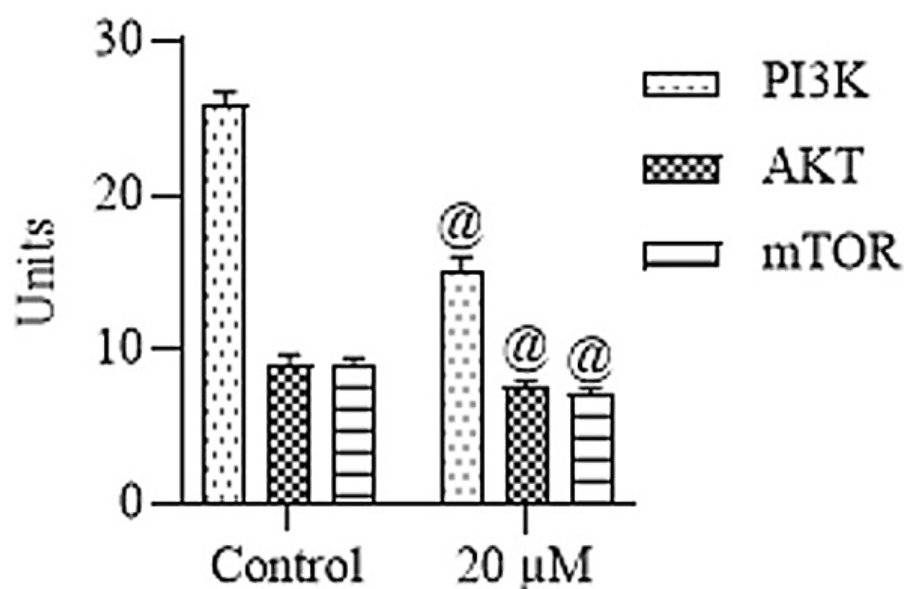


Figure 3: Effect of senegenin on the PI3K/Akt/mTOR protein levels in the A549 cells. The findings are presented as Mean $\pm$ SD of triplicate assays, with each value analyzed using one-way ANOVA and Tukey's *post hoc* assay. "@" indicates that the data in treatment group significantly differ from the control group at $p < 0.05$.

Table 1: List of targets, PDB ID, binding affinity (kcal/mol), RMSD (Å), and their interacted residues with senegenin using different types of bonds.

Targets Name	Targets (PDB ID)	Binding affinity (Kcal/mol)	RMSD (Å)	Hydrogen bonds	Hydrophobic residues	Other types of bonds
PI3K	6ZAD	-7.4	2.263	ALA456, THR458	TRP420, VAL445, ASN454, GLY457, LYS620, TYR621, VAL657, ARG982, ALA983, GLY985, SER990, CYS991	ALA456A
Akt	6CCY	-9.0	2.493	LYS158, GLU191, GLU198	GLY157, GLY159, GLY162, LYS163, LYS179, LEU181, ILE186, HIS194, GLU234, GLU278, ASN279, ASP292, GLY294, LEU295	VAL164A(2), PHE161PS
mTOR	4DRI	-7.4	2.613	ASP2102	TYR2038, VAL2094, THR2098	TRP2101PA, TYR2105PS, PHE2039PA

Note: A -Alkyl; PS -Pi-sigma; PA - Pi-alkyl.

Additionally, LigPlot+ confirmed the interactions between PI3K, Akt, and mTOR with senegenin. The hydrogen bonds were formed between senegenin and the PI3K residues THR458 and SER990, the Akt residues GLU198 and GLU191, and the mTOR residues TYR2038 and ASP2102 (Figures 4G-4I).

Drug-likeness and Toxicity prediction

Senegenin exhibited drug-likeness properties that includes acceptable molecular weight 537.13 g/mol, six and four hydrogen bond acceptors and donors. Also, the Topological Polar Surface Area (TPSA) was observed as 115.06 Å² and the estimated LogP value was 5.49 which indicate moderate lipophilicity (Table 2). Together these parameters properties suggest the senegenin's potential oral bioavailability.

The toxicity profile revealed that senegenin is generally safe with low to mild predicted toxicities across different endpoints with the lethal dose of 6176 mg/kg (Table 2). It showed negative predictions for hepatotoxicity (0.94), neurotoxicity (0.93), mutagenicity (0.86), cytotoxicity (0.80), and ecotoxicity (0.64). Mild activity was observed for nephrotoxicity (0.50), cardiotoxicity (0.68), carcinogenicity (0.51), and clinical toxicity (0.66). Positive predictions were noted for respiratory toxicity (0.82), immunotoxicity (0.86), and nutritional toxicity (0.71). The compound had a high prediction probability of crossing the blood–brain barrier (0.74) indicating possible Central Nervous System (CNS) activity. Based on the overall senegenin profile was assigned a toxicity class VI indicating low risk of toxicity.

DISCUSSION

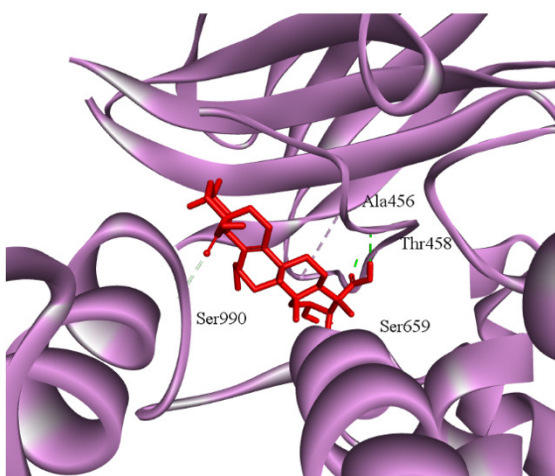
Lung cancer continues to be a major global health concern, necessitating innovative approaches to improve therapeutic outcomes. Despite significant advancements in conventional therapies, including chemotherapy, radiotherapy, and targeted therapy, the disease remains a foremost cause of cancer-related

deaths globally.²³ This is largely due to late-stage diagnoses, as symptoms often manifest when the disease has already metastasized, particularly to lymph nodes and other organs. The five-year survival rate for lung cancer remains low, hovering around 16%, primarily due to these delayed identifications. Current treatment modalities, while effective in some cases, often present substantial burdens, including severe side effects and limited efficacy against aggressive forms of the disease.²⁴ These limitations highlight the critical need for alternative therapies that can address drug resistance, reduce treatment costs, and minimize adverse effects associated with existing protocols. The lack of early symptoms in lung cancer often results in diagnosis at advanced stages, making effective treatment challenging and underscoring the urgent need for novel interventions. Moreover, the inherent heterogeneity within lung tumors and the complex biological characteristics of cancer cells further complicate treatment efforts, resulting in drug resistance and recurrence.²⁵ The development of effective treatments is further complicated by the fact that nearly half of all cancer cells are detected in stage 4, where the survival rate is less than 6%. This necessitates a shift towards complementary therapeutic strategies, particularly those incorporating phytochemicals, which offer multi-targeted effects, lower toxicity, and cost-effectiveness.²⁶

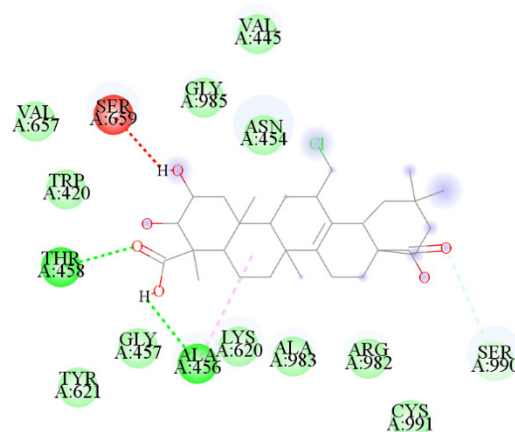
In the context of cancer research, evaluating the cytotoxic potential of novel drug candidates against various cancer cell lines is a critical step in preclinical drug development, enabling the identification of compounds that selectively induce cell death in malignant cells while sparing healthy tissues. This rigorous assessment is paramount for progressing promising agents through the complex and multi-stage process of drug discovery and development.²⁷ This initial screening helps to identify the therapeutic window of a compound, providing insights into its efficacy and potential toxicity profile before proceeding to more complex and costly in vivo studies. The utilization of cell viability

PI3K (PDB ID: 6ZAD)

(A)

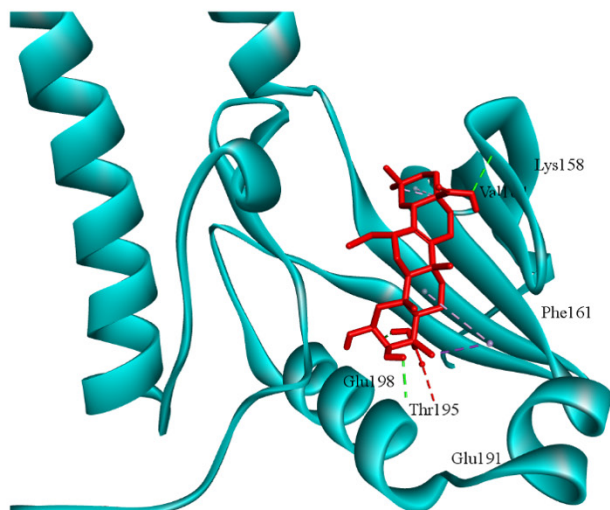


(B)

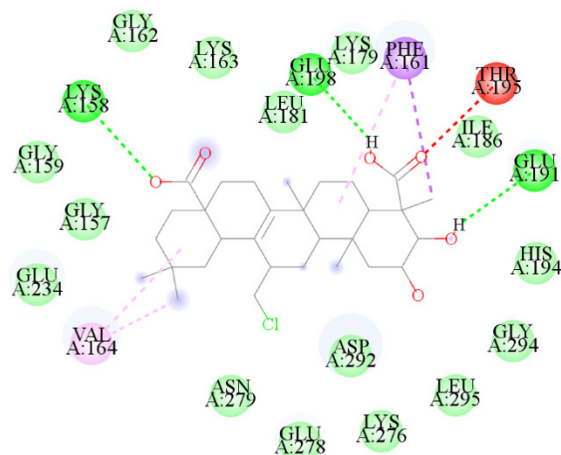


AKT1 (PDB ID: 6CCY)

(C)

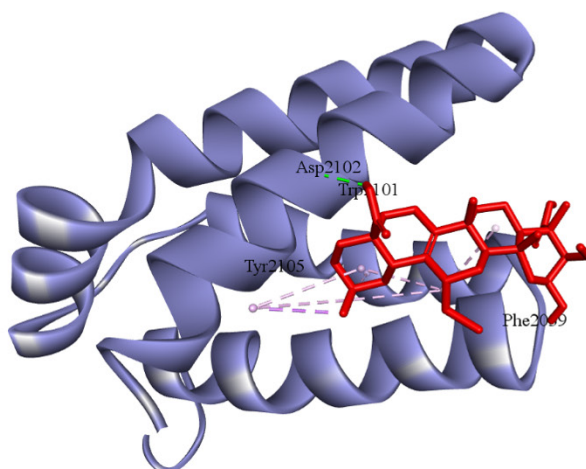


(D)

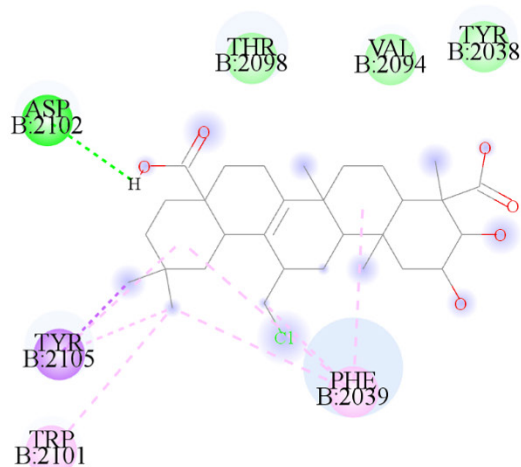


mTOR (PDB ID: 4DRI)

(E)



(F)



Interactions Type

- Conventional hydrogen bond
- Pi-sigma
- Pi-alkyl
- Alkyl

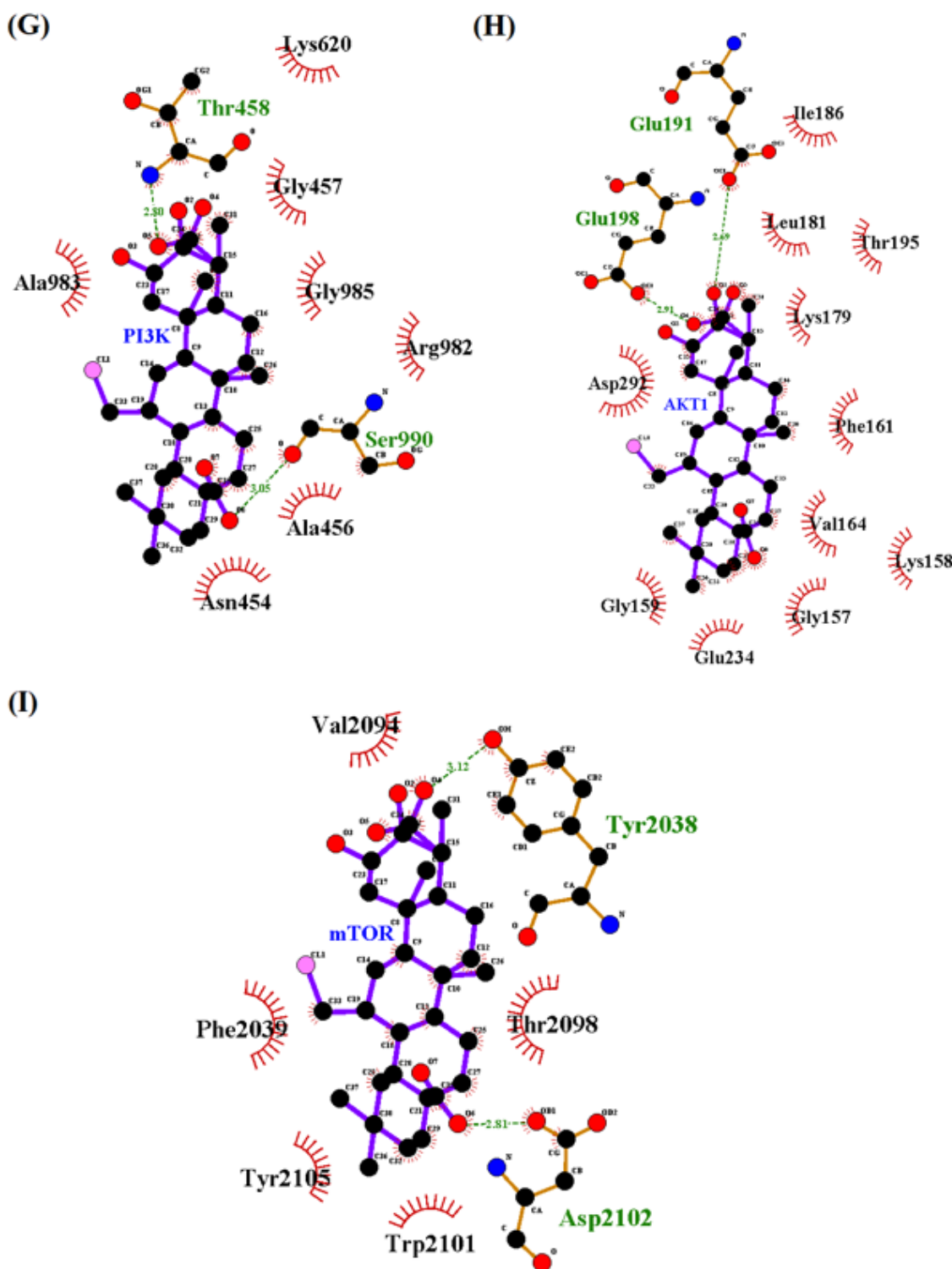


Figure 4: The docking poses of targets PI3K, Akt, and mTOR with the senegenin and its validation using LigPlot+. (A, B) The 3D and 2D docked poses of the PI3K with senegenin. (C, D) The 3D and 2D docked poses of the Akt with senegenin. (E, F) The 3D and 2D docked poses of the mTOR with senegenin. (G, H, I) The LigPlot+ validation for the PI3K, Akt, mTOR complex with the senegenin to confirm the hydrogen bonds.

assays, for instance, is fundamental in cancer research to evaluate the efficacy of both conventional and novel treatments across various tumor profiles.²⁸ This early-stage cytotoxicity profiling is essential for prioritizing molecules with minimal adverse effects for further evaluation, thereby increasing the likelihood

of successful drug candidates. The analysis of a drug's toxicity is a primary task in clinical pharmacology, where a decrease in tumor cell viability can indicate either inhibited proliferation due to metabolic disruptions or actual cell death. This early-stage evaluation is crucial for distinguishing between desired apoptotic

Table 2: Drug likeness and toxicity profile for the senegenin.

Parameters	Probability
Drug Likeness	
Molweight	537.13
Number of hydrogen bond acceptors	6
Number of hydrogen bond donors	4
Topological Polar Surface Area	115.06
octanol/water partition coefficient (logP)	5.49
Toxicity	
Hepatotoxicity	0.94 (-)
Neurotoxicity	0.93 (-)
Nephrotoxicity	0.50 (Mild active)
Respiratory toxicity	0.82 (+)
Cardiotoxicity	0.68 (Mild active)
Carcinogenicity	0.51 (Mild active)
Immunotoxicity	0.86 (+)
Mutagenicity	0.86 (-)
Cytotoxicity	0.8 (-)
BBB-barrier	0.74 (+)
Ecotoxicity	0.64 (-)
Clinical toxicity	0.66 (Mild active)
Nutritional toxicity	0.71 (+)
LD ₅₀	6176 mg/kg
Toxicity class	VI

cell death and undesirable necrotic cell death, a key factor in assessing true anticancer potential.²⁹ Indeed, drug toxicity is a major factor contributing to the high attrition rate of drug candidates, accounting for approximately one-third of all failures during development. This underscores the critical importance of robust preclinical platforms for screening antitumor compounds, especially given the low clinical approval rate of approximately 7% for anticancer drugs. Therefore, comprehensive *in vitro* cytotoxicity assessments are vital for predicting the *in vivo* toxicology of drug candidates and minimizing potential health and environmental risks.³⁰ The MTT test is a colorimetric method broadly utilized for assessing cell viability and cytotoxicity of drug compounds against various cancer cell lines. This assay represents a common approach in initial anticancer drug development due to its relative simplicity and high-throughput compatibility. This makes the MTT assay a robust tool for evaluating inhibitory effects of compounds on cell growth.³¹ In the present study, the MTT assay was utilized to assess the cytotoxicity of senegenin against lung cancer cells. The findings of the MTT assay revealed a considerable decrease in the growth of lung cancer A549 cells

after exposure to different dosages of senegenin. These results highlight that senegenin can inhibit the viability of lung cancer cells by its cytotoxicity.

Apoptosis, a finely regulated physiological process of programmed cell death, plays a critical role in sustaining tissue homeostasis and discarding aberrant cells, thereby acting as a natural defense mechanism against tumorigenesis.³² The dysregulation of apoptotic pathways is a hallmark of cancer, permitting cancer cells to escape apoptosis and proliferate uncontrollably. This evasion of apoptosis, often due to alterations that block cell death signaling pathways, allows for the accumulation of mutations, promotes angiogenesis, and increases invasiveness during tumor progression.³³ Consequently, many anti-cancer therapies aim to reactivate or induce apoptosis in malignant cells to effectively eliminate them. This therapeutic strategy involves targeting specific molecular components within the apoptotic machinery, thereby overcoming the resistance mechanisms developed by cancer cells. Understanding the intricate interplay between various signaling pathways that converge on apoptosis is crucial for developing novel therapeutic agents.³⁴ Specifically, natural compounds often induce apoptosis through diverse mechanisms, including the mitochondrial (intrinsic) pathway and the death receptor (extrinsic) pathway, both critical for eradicating unwanted cells. Many anti-cancer natural products achieve this by modulating key apoptotic proteins involved in these pathways.³⁵

In the context of cancer, dysregulation of apoptosis often contributes to tumor development and resistance to therapy, making its induction a primary goal for many anticancer drugs. Consequently, accurate and reliable methods for detecting and quantifying apoptosis are indispensable for evaluating the efficacy of novel therapeutic compounds. One such method is the dual staining assay utilizing AO and EB, which differentiates viable, apoptotic, and necrotic cells based on membrane integrity and nuclear morphology.³⁶ This differential staining provides a rapid and cost-effective approach to distinguish desirable programmed cell death from non-specific necrosis, which is crucial in early-stage drug evaluation. This makes it a valuable tool for assessing the mechanisms of action of potential anticancer agents. Further, the assay aids in deciphering the dose-response relationship of experimental drugs, providing critical insights into their therapeutic windows and potential toxicity.³⁷ In this work, the level of apoptotic incidences in untreated and senegenin-treated lung cancer cells was evaluated using a dual staining assay. The present results indicated that senegenin treatment considerably increased the apoptotic incidences in the A549 cells. Therefore, it can be suggested that senegenin treatment can inhibit the lung cancer progression via inducing apoptosis in lung cancer cells.

The PI3K/Akt/mTOR pathway is a crucial intracellular signaling axis that controls numerous cellular mechanisms vital for cell survival and proliferation. Dysregulation within this pathway is frequently observed in human cancers, contributing significantly

to tumorigenesis and resistance to various anticancer therapies.³⁸ Specifically, over activation of PI3K, loss of function of the tumor suppressor PTEN, and gain-of-function mutations in Akt are notorious drivers of disease progression and treatment evasion in numerous malignancies. This aberrant activation, recognized as the most often dysregulated pathway in cancers, fosters an environment conducive to uncontrolled cell division, inhibited apoptosis, and enhanced angiogenesis, thereby fueling tumor growth and metastasis.^{39,40} Furthermore, the PI3K/Akt/mTOR cascade exerts significant influence on the tumor microenvironment by promoting angiogenesis and recruiting inflammatory factors, further accelerating cancer progression. The intricate cross-talk between the PI3K/Akt/mTOR axis and other pathways further complicates its role in oncogenesis, as these interactions can either potentiate or mitigate its effects on cellular functions.⁴¹

Given its pervasive involvement in oncogenesis, the PI3K/Akt/mTOR axis represents a crucial therapeutic target for developing novel anticancer strategies. This has led to extensive research into inhibitors targeting various components of this cascade, aiming to counteract the uncontrolled cell proliferation and survival characteristic of cancer.^{42,43} The PI3K/Akt/mTOR axis regulates numerous cellular functions, including proliferation and metabolism. This intricate pathway is also deeply interconnected with other crucial cellular signaling cascades, such as those involving hypoxia-inducible factors, further complicating its role.⁴⁴ Consequently, the sustained activation of the PI3K/Akt/mTOR axis fosters an environment conducive to tumorigenesis, influencing hallmarks such as apoptosis, angiogenesis, and chemoresistance. Therefore, effective targeting of this signaling network with pharmacological inhibitors could lead to more efficacious and less toxic treatments for cancer patients.⁴⁵ In this, the *in vitro* analysis, revealed that the senegenin treatment markedly decreased the PI3K, Akt, and mTOR levels in the A549 cells. These outcomes highlight that senegenin treatment can inhibit the lung cancer progression via down-regulating the PI3K/Akt/mTOR axis.

The molecular docking analysis additionally revealed that senegenin significantly interacted with the PI3K, Akt, and mTOR based on the hydrogen bond formation and RMSD ($\leq 3.0\text{\AA}$) which confirmed that senegenin could act as a multi-target inhibitor and further prevent the downstream signaling event associated with the lung cancer progression. Also, the predicted drug-likeness and toxicity profile revealed that the senegenin under low toxicity risk and could be included in the treatment of lung cancer after confirmed with extensive *in vitro* and *in vivo* experiments.

The present study has several limitations, such as we did not perform additional assays like LDH or Annexin V to confirm cytotoxic effects. We measured total PI3K, Akt, and mTOR levels but not their active phosphorylated forms. Molecular

docking analysis lacked re-docking of co-crystallized ligands and comparison with known inhibitors. The study focused on A549 cells, limiting broader applicability. Senegenin's effects on other lung cancer cell lines and *in vivo* models were not explored. Protein expression changes were not validated at the mRNA level. Downstream effectors of the PI3K/Akt/mTOR pathway were not examined. These limitations will be addressed in our future studies to further elucidate senegenin's anticancer mechanisms and potential therapeutic applications.

CONCLUSION

In conclusion, the current findings indicate that senegenin treatment reduces reduced cell metabolic viability and enhances apoptosis in A549 cells. Senegenin treatment enhanced apoptotic incidences and down-regulated the PI3K/Akt/mTOR axis in A549 cells. Also, the senegenin exhibits strong binding interactions with key regulators of the PI3K/Akt/mTOR signaling pathway, indicating its potential role as a multi-target inhibitor in lung cancer therapy. Altogether, these results found that senegenin may serve as a promising anticancer candidate for the possible treatment of lung cancer. Nevertheless, further studies are necessary to understand the molecular mechanisms underlying senegenin-induced apoptosis in lung cancer cells, which will facilitate the development of a viable anticancer agent to treat lung cancer. Moreover, extensive *in vitro* and *in vivo* investigations are warranted to validate its clinical applicability and therapeutic efficacy.

ABBREVIATIONS

PI3K: Phosphatidylinositol 3-kinase; **AKT:** Protein kinase B; **mTOR:** Mammalian target of rapamycin; **AO:** Acridine orange; **EB:** Ethidium bromide; **PDB:** Protein Data Bank. **MTT:** 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **DSV:** Discovery Studio Visualizer; **BA:** Binding affinity; **RMSD:** Root Mean Square Deviation; **ADMET:** Absorption, Distribution, Metabolism, Excretion, Toxicity; **3D:** 3-dimensional; **DMEM:** Dulbecco's Modified Eagle Medium; **FBS:** Fetal Bovine Serum; **DMSO:** Dimethyl Sulfoxide; **SD:** Standard Deviation; **ANOVA:** Analysis of Variance; **IC₅₀:** Half Maximal Inhibitory Concentration; **PDBQT:** Protein Data Bank, Partial Charge (Q), and Atom Type (T); **TPSA:** Topological Polar Surface Area; **LogP:** Octanol/water partition coefficient; **LD₅₀:** Lethal Dose, 50%; **BBB-barrier:** Blood-brain barrier; **CNS:** Central nervous system; **A:** Alkyl; **PS:** Pi-sigma; **PA:** Pi-alkyl.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

Xinxiang Xu: Conceptualization, Writing Original Draft, Data Analysis, and Review and Editing.

SUMMARY

Lung cancer continues to be a primary contributor to cancer-related mortalities, largely due to its significant intratumor heterogeneity and the complex cellular characteristics inherent to the disease. The challenges associated with current therapies, such as systemic toxicities and the onset of drug resistance, necessitate the exploration of novel therapeutic avenues with enhanced efficacy and reduced adverse effects. The current findings indicate that senegenin treatment reduces cell viability and enhances apoptosis in A549 cells via down-regulating PI3K/Akt/mTOR axis. These results found that senegenin may serve as a promising anticancer candidate for the possible treatment of lung cancer.

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