

Nuclear-Targeted Engineering of a Lunasin-Derived Peptide Enhances Antiproliferative Activity and AP-1-Associated Gene Modulation in A549 Lung Cancer Cells

Zahraa Amer Al-Juboori^{*1}  , Inam Sameh Arif¹  , Ayman M. Albanna²  ,
Israa Al-Banaa³  , Md. Abdullah-Al-Mamun Sarker^{**4}  

¹ Department of Pharmacology and Toxicology, College of Pharmacy, Mustansiriyah University, Baghdad, Iraq

² Department of Veterinary Public Health, College of Veterinary Medicine, University of Mosul, Mosul, Iraq

³ School of Biomedical Sciences, Faculty of Biological Sciences, University of Leeds, Leeds, United Kingdom

⁴ Department of Biochemistry and Molecular Biology, Shahjalal University of Science and Technology, Sylhet, Bangladesh

*Corresponding Author¹ email: Zahraa_ammer@uomustansiriyah.edu.iq

ORCID: <https://orcid.org/0000-0002-2115-2965>

**Corresponding Author² email: almamun.sust98@gmail.com

ORCID: <https://orcid.org/0009-0007-8533-1122>

DOI: <https://doi.org/10.32947/ajps.v26i3.1404>

Received 19/May/2026, Revised 15/July/2026, Accepted 16/Aug/2026, Published 30/Sept/2026

Abstract:

Activator protein-1 (AP-1) is a transcriptional regulator involved in cancer-associated processes, including proliferation, invasion, and angiogenesis; however, its broad protein–DNA interaction interface limits direct targeting by conventional small molecules. This study aimed to evaluate whether nuclear-targeted engineering of a previously designed lunasin-derived peptide could enhance its antiproliferative and gene-regulatory activity in A549 lung adenocarcinoma cells. We hypothesized that the modified nuclear localization signal (NLS)-containing peptide would show stronger biological activity than the native/core peptide sequence. Two peptide forms were compared: Compound A, a 27-amino acid modified peptide containing a rigid EAAAK linker and an NLS motif, and Compound B, the native/core peptide. STRING and Cytoscape-based network analysis supported the selection of AP-1-associated downstream genes, including MMP9, CCND1, and VEGFA, for experimental validation. Importin- α docking suggested that Compound A could be accommodated within the NLS-recognition region and displayed broader receptor engagement than Compound B, with direct involvement of NLS-region residues. In A549 cells, both compounds reduced cell viability in a time- and concentration-dependent manner; however, Compound A showed consistently stronger antiproliferative activity, with IC₅₀ values of 52.69, 37.35, and 18.60 μ M after 24, 48, and 72 h, respectively, compared with 238.26, 81.42, and 43.34 μ M for Compound B. Microscopic examination further showed reduced cell density and treatment-associated morphological alterations. Exploratory RT-qPCR analysis showed that Compound A reduced MMP9 and VEGFA expression and produced a marked decreasing trend in CCND1 expression. In contrast,

AJPS (2026)

331



Compound B showed a non-significant reduction in MMP9 but non-significant increases in CCND1 and VEGFA, indicating a variable and non-suppressive transcriptional pattern. These findings suggest that nuclear-targeted modification enhanced the biological activity of the lunasin-derived peptide in A549 cells. Although direct mechanistic confirmation of AP-1 inhibition remains required, this study provides preliminary in vitro evidence supporting Compound A as a promising peptide candidate for further investigation.

Keywords: AP-1 transcription factor; A549 cells; lunasin-derived peptide; nuclear localization signal; importin- α ; MTT assay; RT-qPCR; MMP9; CCND1; VEGFA; non-small cell lung cancer.

Introduction

Lung cancer remains one of the leading causes of cancer-related mortality worldwide, accounting for approximately 1.8 million deaths in 2022 (1). Non-small cell lung cancer (NSCLC) represents the major histological category of lung cancer and is characterized by substantial molecular heterogeneity, variable therapeutic response, and frequent development of resistance to chemotherapy, targeted therapy, and immunotherapy (2). These challenges highlight the need for additional therapeutic strategies that target intracellular regulatory mechanisms controlling tumor-associated gene-expression programs, including transcriptional networks involved in proliferation, survival, invasion, and angiogenesis (3).

Activator protein-1 (AP-1) is a dimeric transcription factor complex mainly formed by members of the JUN and FOS protein families (4,5). AP-1 integrates signals from multiple upstream pathways, including mitogenic, inflammatory, stress-related, and growth factor-associated signaling cascades, and regulates genes involved in proliferation, survival, invasion, inflammation, angiogenesis, and cellular adaptation (4,5). Because of this regulatory role, AP-1 has been considered a biologically relevant transcriptional framework in cancer biology, including lung cancer (3–5).

Despite its biological relevance, AP-1 remains challenging to target directly. Like many transcription factors, AP-1 lacks a deep

enzymatic pocket and functions through broad protein–DNA and protein–protein interaction interfaces, which limits the applicability of many conventional small-molecule strategies (6). Peptide-based approaches may provide an alternative strategy for modulating such interfaces, because peptides can present larger and more flexible interaction surfaces capable of engaging extended or shallow binding regions (7).

In previous computational work, the lunasin-derived decapeptide sequence SKWQHQQDSC was prioritized as a candidate AP-1-interacting peptide. Further in silico engineering suggested that linking this core sequence to a nuclear localization signal (NLS) through a rigid EAAAK-based linker could preserve the predicted AP-1-interacting region while adding a nuclear-targeting feature (8). This modification was biologically rational because AP-1 functions mainly within the nuclear compartment; therefore, a peptide intended to influence AP-1-associated transcriptional outputs should be compatible with intracellular and nuclear-directed delivery.

Classical NLS-containing cargos are recognized through the importin-mediated nuclear import pathway. Importin- α acts as an adaptor that recognizes basic NLS motifs and supports nuclear translocation through the importin- α /importin- β transport system (9,10). Accordingly, docking the modified peptide against importin- α provides supportive structural information regarding



the compatibility of the appended NLS segment with a nuclear-import recognition pathway. In parallel, network-based bioinformatics analysis using STRING and Cytoscape can place AP-1-associated proteins within a broader functional interaction network and support the selection of downstream genes for experimental validation (11,12).

Accordingly, the present study was designed to move from computational peptide engineering toward early in vitro validation. Two peptide forms were compared in A549 lung adenocarcinoma cells: Compound A, the modified 27-amino acid peptide containing the AP-1-interacting core sequence, rigid EAAAK linker, and NLS motif; and Compound B, the native/core peptide sequence. We hypothesized that the NLS-modified peptide would demonstrate stronger antiproliferative and AP-1-associated gene-regulatory activity than the native/core peptide. To test this hypothesis, the study combined network-guided gene selection, importin- α docking, MTT-based cell viability assessment, microscopic examination, and RT-qPCR analysis of selected AP-1-associated downstream genes, including MMP9, CCND1, and VEGFA.

Materials

The human lung adenocarcinoma cell line A549 was used as the in vitro experimental model. A549 cells were obtained from the American Type Culture Collection (ATCC, USA). Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Capricorn Scientific, Germany) supplemented with 10% (v/v) fetal bovine serum (FBS; Capricorn Scientific, Germany) and 1% (v/v) penicillin–streptomycin solution (100 $\times$; Capricorn Scientific, Germany). Phosphate-buffered saline (PBS; Capricorn Scientific, Germany) was used for cell washing and MTT-solution preparation. Trypsin–EDTA 0.25% was used for routine

detachment and subculturing of adherent cells.

The tested peptides were commercially synthesized by GenScript, China, and supplied in lyophilized form. Compound A was the modified 27-amino-acid peptide containing the native/core sequence, a double EAAAK rigid linker, and a nuclear localization signal (NLS), with the sequence SKWQHQQDSCEAAAKEAAAKPKKKR KV. Compound B was the native/core 10-amino-acid peptide with the sequence SKWQHQQDSC. Compound A was supplied as a TFA salt with N-terminal acetylation and C-terminal amidation. Manufacturer-provided quality-control analysis showed a theoretical molecular weight of 3093.57 Da, observed molecular weight by mass spectrometry of 3094.2 Da, HPLC purity of 95.1%, HPLC major peak area of 95.077%, LC-MS main peak area of 95.24%, and net peptide content of 75.83%. The lyophilized peptide was stored at -20°C until use.

For the MTT assay, MTT reagent was obtained from a commercial laboratory supplier and prepared in PBS before use. Dimethyl sulfoxide (DMSO) was used to dissolve the formed formazan crystals. Peptide stock solutions were prepared under sterile conditions, and working concentrations were freshly prepared by dilution in complete culture medium immediately before treatment.

For RT-qPCR analysis, total RNA was extracted using TRIzol reagent and chloroform. RNase-free water was obtained from Santa Cruz Biotechnology, USA. Complementary DNA was synthesized using EasyScript® One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech, China). Quantitative real-time PCR was performed using a SYBR Green-based qPCR SuperMix (TransGen Biotech, China). Gene-specific primers for MMP9, CCND1,



VEGFA, and GAPDH were used, with GAPDH serving as the internal housekeeping gene. Primer sequences are provided in Table 1.

Sterile tissue-culture consumables, including culture flasks, flat-bottomed 96-well plates, 6-well plates, centrifuge tubes, sterile pipette tips, coverslips, and glass slides, were obtained from commercial laboratory suppliers. The main laboratory instruments used in this study included a humidified CO₂ incubator for cell culture, a Class II biological safety cabinet for aseptic handling, an inverted light microscope for monitoring cell growth and morphology, a microplate reader for MTT absorbance measurement, a NanoDrop spectrophotometer for RNA concentration and purity assessment, and a real-time PCR system for SYBR Green-based quantitative PCR analysis.

The computational part of the study used Schrödinger Suite (Schrödinger LLC, New York, NY, USA), including Maestro, Protein Preparation Wizard, and BioLuminate/PIPER for peptide and protein preparation, docking, and interaction analysis. The RCSB Protein Data Bank was used to retrieve the importin- α structure and was accessed on 03 January 2026. GEPIA2 was used for tumor-normal expression comparison and survival analysis and was accessed on 03 January 2026. The STRING database was used for protein-protein interaction analysis and was accessed on 10 October 2025. Cytoscape version 3.10 was used for network visualization and interpretation.

Methodology

Study design

This study was designed to evaluate whether nuclear-targeted modification of a previously designed lunasin-derived peptide enhances its biological activity in A549 lung adenocarcinoma cells. The workflow included bioinformatics-based target and

gene selection, in silico assessment of importin- α recognition, peptide synthesis and preparation, MTT-based cell viability analysis, morphological examination, and RT-qPCR analysis of selected AP-1-associated downstream genes.

Bioinformatics-based network analysis and gene selection

A bioinformatics-guided workflow was used to support the selection of AP-1-associated transcriptional signaling and to identify downstream genes for experimental validation. The AP-1-associated protein network was generated using the STRING database, which integrates known and predicted protein-protein associations from experimental, curated, computational, and text-mining sources (11). The resulting interaction network was imported into Cytoscape version 3.10 for visualization and network interpretation (12).

Functional enrichment and gene-target relationship analyses were used to support the selection of downstream genes linked to AP-1-associated cancer biology. The selected genes for RT-qPCR validation were MMP9, CCND1, and VEGFA, representing extracellular matrix remodeling/invasion, cell-cycle progression, and angiogenesis, respectively. GAPDH was used as the internal housekeeping gene for normalization.

In silico assessment of importin- α recognition

To evaluate whether the nuclear localization signal-containing peptide could be structurally accommodated by importin- α , docking analysis was performed using Schrödinger BioLuminate/PIPER. The crystal structure of importin- α was retrieved from the RCSB Protein Data Bank using PDB ID 6BW9 (13). The receptor structure was prepared using the Protein Preparation Wizard, including hydrogen atom addition,



bond-order assignment, protonation-state optimization, hydrogen-bond network optimization, and restrained energy minimization using the OPLS4 force field (14,15).

The modified peptide, Compound A, was docked against the NLS-recognition groove of importin- α . Docking outputs were assessed using PIPER cluster size, pose energy, pose score, and interaction profile. PIPER-based docking was used because it is suitable for protein–protein and peptide–protein docking through sampling of possible binding orientations and clustering of recurrent poses (16). The analysis focused on whether the PKKKRKV NLS motif was oriented toward the importin- α recognition region and whether lysine/arginine residues contributed to receptor interaction. Docking results were interpreted as supportive structural evidence for nuclear-targeting compatibility, not as experimental proof of nuclear import.

Peptide synthesis and preparation

Two peptide forms were evaluated experimentally. Compound A was the modified 27-amino-acid peptide containing the AP-1-interacting core sequence, a rigid double EAAAK linker, and a nuclear localization signal. Compound B was the native/core 10-amino-acid peptide.

Compound A:

SKWQHQQDSCEAAAKEAAAKPKKKR
KV

Compound B: SKWQHQQDSC

Both peptides were commercially synthesized by GenScript and supplied in lyophilized form. Peptide stock solutions were prepared under sterile conditions, and working concentrations were freshly prepared by dilution in complete culture medium immediately before treatment.

Cell culture

Human A549 lung adenocarcinoma cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) penicillin–streptomycin. Cells were maintained at 37°C in a humidified incubator containing 5% CO₂. The culture medium was replaced every two days, and cells were subcultured when they reached approximately 70–80% confluency.

For subculturing, cells were washed with phosphate-buffered saline (PBS) and detached using 0.25% trypsin–EDTA. Trypsin activity was neutralized using complete culture medium. Only healthy, exponentially growing cells were used for experimental treatments.

MTT cell viability assay

The antiproliferative effects of Compound A and Compound B on A549 cells were evaluated using the MTT colorimetric assay. The MTT assay was selected as a standard colorimetric method for estimating viable metabolically active cells based on the reduction of tetrazolium salt into formazan crystals (17,18). A549 cells were seeded into flat-bottomed 96-well tissue-culture plates at a density of 5×10^3 cells/well in 100 μ L complete culture medium and incubated for 24 h under standard culture conditions to allow cell attachment.

After attachment, the culture medium was removed and replaced with fresh complete medium containing serial concentrations of Compound A or Compound B. The tested concentrations were 10.93, 21.8, 43.75, 87.5, 175, 350, and 700 μ M. Untreated cells were included as the negative control. Each concentration was tested in triplicate, and plates were incubated for 24, 48, and 72 h at 37°C in a humidified incubator containing 5% CO₂.

At the end of each treatment period, the culture medium was removed and replaced with MTT solution prepared in PBS. The



plates were incubated for 4 h at 37°C to allow metabolically active cells to reduce MTT into purple formazan crystals. After incubation, the MTT solution was carefully removed, and the formed formazan crystals were dissolved by adding 100 µL DMSO to each well. Plates were gently shaken and kept in the dark at room temperature for 15–20 min to ensure complete solubilization.

Absorbance was measured using a microplate reader at 570 nm, with a reference wavelength of 630–650 nm when available. Blank wells containing medium without cells were used for background correction. Cell viability was calculated relative to untreated control cells using the following equation: Cell viability (%) = (Mean OD of treated cells / Mean OD of untreated control cells) × 100

Dose–response curves were generated by plotting cell viability percentage against log-transformed peptide concentration. The half-maximal inhibitory concentration (IC₅₀) was calculated for each compound at each time point using nonlinear regression curve fitting.

Morphological examination

Morphological changes in A549 cells were examined using an inverted light microscope after peptide treatment. Untreated cells were used as the reference control. Treated cells were inspected for visible changes in cell density, cell shape, adherence, shrinkage, rounding, and detachment. Representative microscopic images were captured after treatment and used as qualitative supportive evidence alongside the MTT assay. This examination was not used to define the exact mechanism of cell death.

RNA extraction and cDNA synthesis

For RT-qPCR analysis, A549 cells were treated with Compound A or Compound B at concentrations approximating their respective 24 h IC₅₀ values. Compound A was used at 52.69 µM, whereas Compound B was

used at 238.26 µM. Untreated cells maintained under the same culture conditions were used as the calibrator control. Cells were exposed to the peptides for 24 h, and each experimental condition was represented by two independent biological replicates.

Following treatment, the culture medium was removed, and the cells were gently washed with PBS to remove residual medium and detached cells. Total RNA was extracted using TRIzol reagent according to the manufacturer's instructions. Briefly, TRIzol reagent was added directly to the cells to induce lysis and preserve RNA integrity. Chloroform was then added to achieve phase separation, and the upper aqueous phase containing RNA was carefully transferred into a new RNase-free tube. RNA was subsequently precipitated, washed, and reconstituted in RNase-free water. All procedures were performed under RNase-free conditions to minimize RNA degradation.

RNA concentration was determined spectrophotometrically by measuring absorbance at 260 nm. RNA purity was assessed using the A260/A280 absorbance ratio, and samples with acceptable RNA concentration and purity were selected for reverse transcription. Complementary DNA was synthesized from equal amounts of total RNA using EasyScript® One-Step gDNA Removal and cDNA Synthesis SuperMix according to the manufacturer's instructions. The synthesized cDNA was stored at –20°C until quantitative real-time PCR analysis.

Quantitative real-time PCR

Quantitative real-time PCR was performed to evaluate the relative expression levels of MMP9, CCND1, and VEGFA following treatment with Compound A or Compound B. GAPDH was used as the internal reference gene. PCR amplification was performed using SYBR Green-based detection on a real-time PCR system. Each reaction contained



SYBR Green qPCR SuperMix, cDNA template, gene-specific forward and reverse primers, and nuclease-free water.

Each qPCR reaction was prepared in a final volume of 20 μ L, containing 10 μ L of 2 $\times$ SYBR Green qPCR SuperMix, 3 μ L cDNA template, 1 μ L forward primer (10 μ M), 1 μ L reverse primer (10 μ M), and 5 μ L nuclease-free water.

PCR amplification was performed using the following thermal programme: initial denaturation at 94°C for 30 s for one cycle,

followed by 40 cycles of denaturation at 94°C for 5 s, annealing at 58–60°C for 15 s, and extension at 72°C for 20 s. An annealing temperature of 58°C was applied for GAPDH, whereas 60°C was applied for the target-gene primer sets. Melt-curve analysis was performed from 55°C to 95°C after amplification to evaluate amplification specificity and identify possible nonspecific amplification or primer-dimer formation. Relative gene expression was calculated using the comparative Ct method (20):

$$\Delta Ct = Ct_{(\text{target gene})} - Ct_{(\text{GAPDH})}$$

$$\Delta\Delta Ct = \Delta Ct_{(\text{treated group})} - \Delta Ct_{(\text{untreated control})}$$

$$\text{Relative fold change} = 2^{-\Delta\Delta Ct}$$

Untreated control cells were assigned a relative fold change of 1.0. Because only two independent biological replicates were used for each condition, the RT-qPCR findings

were interpreted as preliminary gene-expression evidence rather than definitive mechanistic confirmation.

Table 1. Primer sequences used for RT-qPCR analysis

Gene	Forward primer sequence 5'–3'	Reverse primer sequence 5'–3'	Source
MMP9	TTGACAGCGACAAGAAGTGG	GCCATTACGTCGTCCTTAT	Al-Khfajy (21)
CCND1	AAACAGATCATCCGCAAACAC	GTTGGGGCTCCTCAGGTTC	Al-Khfajy (21)
VEGFA	TTGCCTTGCTGCTCTACCTCCA	GATGGCAGTAGCTGCGCTGATA	Sarkar et al. (22)
GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG	Al-Khfajy (21)

Statistical analysis

Quantitative data were expressed as mean $\pm$ standard deviation (SD), unless otherwise stated. For the MTT assay, cell viability was calculated relative to untreated control cells, and IC₅₀ values were obtained using nonlinear dose–response curve fitting. For RT-qPCR analysis, relative gene-expression values were calculated using the 2^{−ΔΔCt} method (20). Statistical comparisons for RT-qPCR were performed using ΔCt values rather than fold-change values. Exploratory comparisons between each peptide-treated group and the untreated control were conducted using an unpaired two-tailed Welch's t-test. A p-value of <0.05 was considered nominally statistically significant. Because RT-qPCR was performed using only

two independent biological replicates, the statistical findings were interpreted cautiously as preliminary evidence.

Results

Network-guided identification of AP-1-associated genes

A bioinformatics-guided analysis was performed to support the selection of AP-1-associated transcriptional signaling and to guide the choice of downstream genes for RT-qPCR validation. Because AP-1 functions as a transcriptional complex rather than as a single isolated protein, the analysis was designed to evaluate both representative AP-1 components and their broader functional interaction context.



The AP-1-associated genes FOS and JUN were first examined in lung adenocarcinoma using the GEPIA2 platform. Expression data from TCGA and GTEx were used to compare lung adenocarcinoma tumor tissues with normal lung tissues. The analysis included 483 LUAD tumor samples and 347 normal lung tissue samples, with expression values presented as $\log_2(\text{TPM} + 1)$. The expression analysis showed altered expression patterns of FOS and JUN in LUAD tumor tissues compared with normal lung tissues; however, overlap between tumor and normal groups was observed. Therefore, these expression findings were interpreted as evidence of AP-1-associated transcriptional dysregulation in LUAD, but not as standalone proof of target validity.

Kaplan–Meier overall survival analysis was then used to examine whether individual FOS or JUN expression was associated with overall survival in LUAD. Patients were divided into high- and low-expression groups according to median gene expression. For FOS, the log-rank p-value was 0.10, with $\text{HR}(\text{high}) = 0.78$. For JUN, the log-rank p-value was 0.86, with $\text{HR}(\text{high}) = 0.97$. These results indicated that high versus low expression of FOS or JUN alone was not significantly associated with overall survival in the analyzed LUAD cohort. Accordingly, AP-1 was not selected on the basis of single-gene survival association. Instead, the target rationale was further evaluated using network connectivity, functional enrichment, and published biological evidence supporting the context-dependent role of AP-1 signaling in cancer-related transcriptional regulation.

To assess AP-1 at the network level, a protein–protein interaction network was generated using STRING and visualized using Cytoscape. The resulting AP-1-associated network contained 11 nodes and 55 edges, with an average node degree of 10 and a local clustering coefficient of 1.0. The number of observed interactions was higher

than the expected number of 15 edges, with a PPI enrichment p-value of 2.11×10^{-15} . These findings indicate that the selected AP-1-associated proteins formed a highly connected and functionally related network rather than a random group of proteins. The network included major AP-1-related proteins, including JUN, FOS, JUNB, JUND, FOSB, FOSL1, and FOSL2, together with transcription- and signaling-associated proteins such as ATF2, ATF3, MAPK8, and EP300.

Functional enrichment analysis further supported the biological relevance of the AP-1-associated network. Enriched biological processes were mainly related to transcriptional regulation, stress response, response to calcium ions, and cellular adaptive responses. Molecular function enrichment included RNA polymerase II-specific DNA-binding transcription activator activity, cis-regulatory region DNA binding, and transcription factor binding. Cellular component enrichment linked the network to nuclear transcriptional machinery, including the AP-1 transcription factor complex, RNA polymerase II transcription regulator complexes, and chromatin-associated structures.

Pathway enrichment analysis showed that AP-1-associated proteins were involved in cancer-related and stress-responsive pathways, including MAPK signaling, IL-17 signaling, TNF signaling, and transcription-associated stress-response pathways. Enrichment of the basic leucine zipper (bZIP) domain further supported the structural relevance of the network, as this domain is characteristic of JUN/FOS AP-1 family members.

Importantly, STRING was not used alone to define the final RT-qPCR gene panel, because STRING primarily represents functional protein associations rather than direct transcription factor–target gene regulation. Therefore, candidate downstream



genes were interpreted in combination with transcription-factor/gene relationship evidence and established cancer-related biological relevance. Based on this integrated rationale, several AP-1-associated genes were considered for downstream experimental validation, including CCND1, MMP9, VEGFA, and IL6. For RT-qPCR analysis, MMP9, CCND1, and VEGFA were

selected as focused readouts because they represent three major cancer-related processes: extracellular matrix remodeling and invasion, cell-cycle progression and proliferation, and angiogenesis, respectively. This focused gene panel was therefore used to evaluate whether peptide treatment was associated with transcriptional changes in AP-1-associated cancer biology.

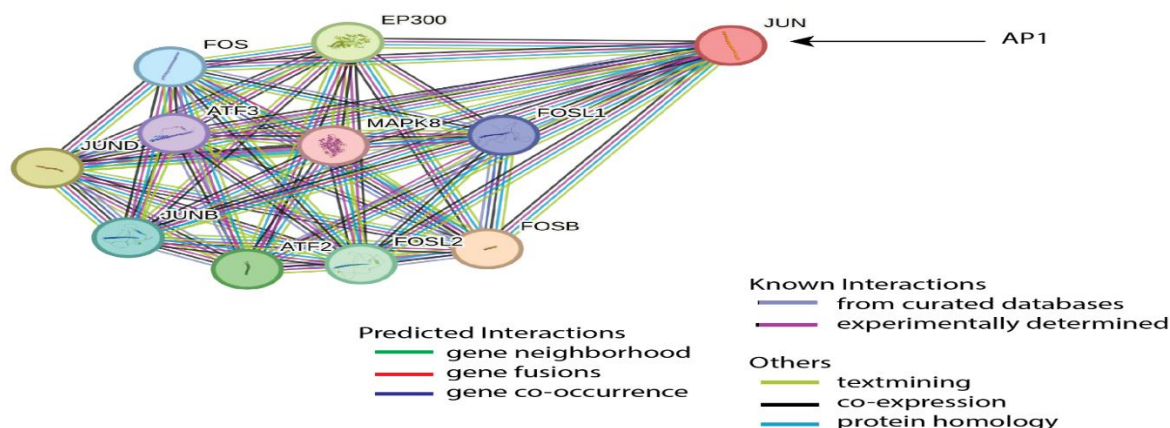


Figure 1. STRING/Cytoscape protein-protein interaction network of AP-1-associated proteins.

The network includes AP-1-related proteins, including JUN, FOS, JUNB, JUND, FOSB, FOSL1, FOSL2, ATF2, ATF3, MAPK8, and EP300. The network contained 11 nodes and 55 edges, with an average node degree of 10 and a clustering coefficient of 1.0. The dense connectivity and significant PPI enrichment support the interpretation of AP-1-associated proteins as a functionally connected transcriptional module.

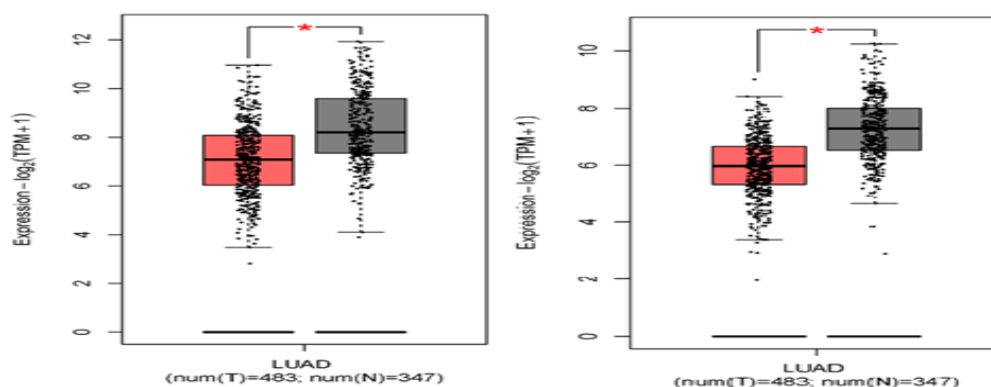


Figure 2. GEPIA2 expression analysis of AP-1-associated components in LUAD. FOS and JUN expression levels were compared between LUAD tumor tissues and normal lung tissues using GEPIA2. Expression values are shown as log₂(TPM + 1), based on 483 LUAD tumor samples and 347 normal lung tissue samples. Kaplan–Meier survival analysis was performed separately and showed no statistically significant overall survival separation for FOS or JUN expression alone.

Importin- α docking analysis

Because Compound A was engineered to contain the classical nuclear localization signal PKKKRKV, docking analysis was performed to examine whether the NLS-containing construct could be accommodated within the importin- α recognition region. This analysis was included to support the nuclear-targeting rationale of the modified peptide, since AP-1 functions as a nuclear transcription factor. However, the docking experiment was interpreted only as an *in-silico* compatibility assessment and not as direct proof of cellular uptake or nuclear import.

Both the native/core peptide and the modified NLS-containing peptide were docked against importin- α using the BioLuminate/PIPER workflow. Compound B represented the native/core AP-1-interacting sequence, whereas Compound A contained the same core sequence linked to a double EAAAK rigid linker and the classical NLS sequence. The docking poses showed that both peptides could be positioned along the importin- α surface groove, but their predicted interaction patterns were different.

The PIPER docking output showed a larger selected cluster for Compound A compared with Compound B. Compound A produced a selected cluster size of 120 poses, whereas Compound B produced a selected cluster size of 15 poses. Compound A also showed lower PIPER pose energy and pose score values than Compound B, with a pose energy of -556.041 and a pose score of -159.283 , compared with -161 and -61.644 for Compound B, respectively. These values were interpreted as comparative docking descriptors under identical docking conditions, with lower energy-like scores considered more favorable. However, because Compound A is longer than Compound B, docking scores were not interpreted alone as proof of stronger binding. Instead, cluster size, pose energy,

pose score, and residue-level interaction patterns were interpreted together.

The interaction profile further indicated that Compound A formed a broader receptor-contact interface than Compound B. Compound B contacted 19 importin- α residues and involved 9 peptide residues, whereas Compound A contacted 25 importin- α residues and involved 18 peptide residues. Although Compound B showed a slightly higher number of hydrogen bonds and marginally higher surface complementarity, Compound A showed features that were more relevant to the nuclear-targeting design, particularly the involvement of residues located within or close to the NLS-containing region.

For Compound A, residues from the NLS-containing region contributed directly to the predicted importin- α interaction. A salt bridge was observed between peptide LYS24 and importin- α ASP326, with additional contacts involving PRO21 and ARG25. These residues are located within or near the NLS-containing segment of the modified construct, suggesting that the appended nuclear-targeting region remained available for receptor engagement in the docking model. In contrast, Compound B lacks the classical PKKKRKV sequence; therefore, its predicted interaction with importin- α was interpreted as a localized surface-contact pattern rather than an NLS-mediated recognition pattern.

The main predicted contacts for Compound B involved importin- α residues TRP348, ARG306, GLU345, ASN310, ASP271, THR313, and GLU387, with hydrogen bonds involving TRP390, LYS386, THR313, and ASP271, together with π - π stacking involving TRP348. For Compound A, the main interacting importin- α residues included HIS252, THR254, HIS253, PRO291, ASP326, ILE214, and PRO210, with a salt bridge between ASP326 and peptide LYS24, hydrogen bonding around



THR254, and π - π stacking involving HIS253.

The docking output also reported van der Waals clashes for both peptides, with 86 clashes for Compound B and 98 clashes for Compound A. These values were not interpreted as favorable interactions. The higher number of clashes in Compound A may partly reflect its larger size and broader contact interface; however, it also indicates that the predicted pose should be interpreted cautiously. Therefore, the docking result supports structural compatibility between the NLS-containing peptide and importin- α , but it does not establish an optimized or experimentally confirmed import complex.

Overall, the importin- α docking analysis supported the design rationale for adding the PKKKRKV nuclear localization sequence to the selected peptide. Compound A showed a larger docking cluster, lower energy-like docking descriptors, broader receptor engagement, and direct participation of NLS-region residues. Nevertheless, because the docking pose also showed a relatively high number of van der Waals clashes, these findings should be considered supportive computational evidence only. Experimental confirmation using confocal co-localization with nuclear staining, quantitative nuclear/cytoplasmic fluorescence analysis, or nuclear/cytoplasmic fractionation would be required to confirm true nuclear accumulation.

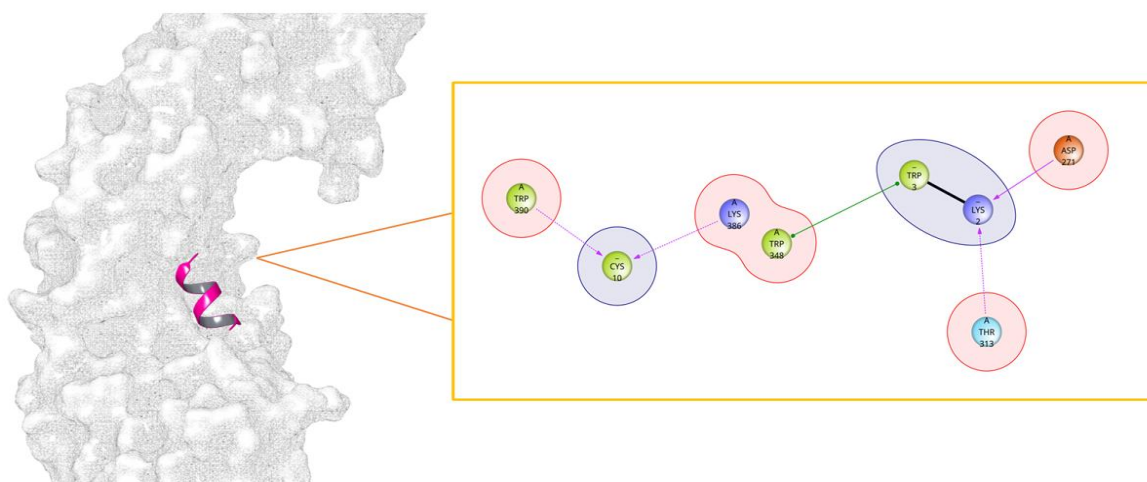


Figure 3. Importin- α docking pose of Compound B. The native peptide was docked against importin- α using the BioLuminate/PIPER workflow. The peptide showed a localized surface-contact pattern along the importin- α groove; however, this interaction was not interpreted as NLS-mediated recognition because Compound B lacks the PKKKRKV nuclear localization sequence.

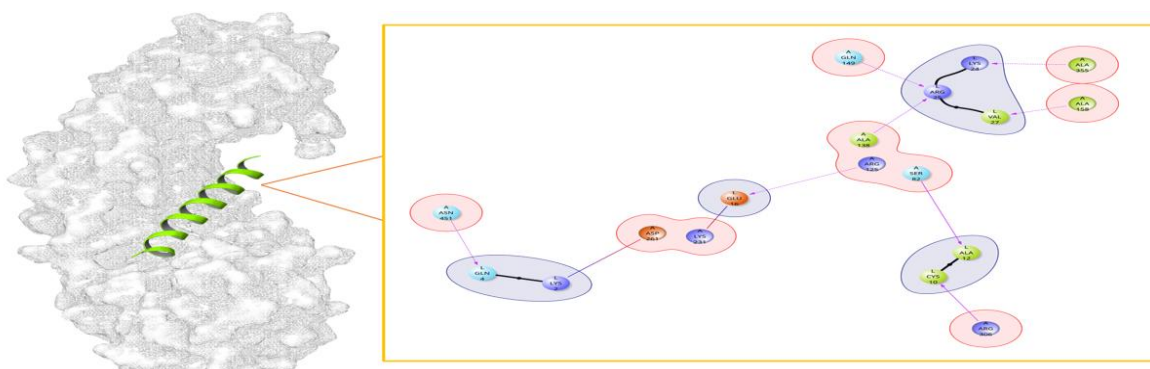


Figure 4. Interaction diagram of Compound A with importin- α . The interaction map shows predicted hydrogen-bonding, electrostatic, aromatic, and surface contacts between Compound A and importin- α . The salt bridge between peptide LYS24 and importin- α ASP326 supports the contribution of the NLS-containing region to receptor engagement. However, this docking output should be interpreted as computational support only, not as confirmation of nuclear transport.

Table 2 Comparative docking interaction profile of the native 10-aa peptide and modified 27-aa peptide with importin- α

Parameter	Native 10-aa peptide	Modified 27-aa peptide, second pose
Peptide sequence/length	SKWQHQQDSC /10 amino acids	SKWQHQQDSCEAAAKEAAKPKKKRK V / 27 amino acids
Selected PIPER cluster size	15	120
PIPER pose energy	-161	-556.041
PIPER pose score	-61.644	-159.283
Total interaction/contact rows	41	57
Number of importin- α residues involved	19	25
Number of peptide residues involved	9	18
Hydrogen bonds	4	2
Salt bridges	0	1
π - π stacking interactions	2	1
Disulfide interactions	0	0
van der Waals clashes reported	86	98
Distance range of recorded contacts	0.6–3.8 Å	0.6–4.0 Å
Maximum surface complementarity	0.82	0.78
Mean surface complementarity	0.4	0.38
Most involved importin- α residues	TRP348, ARG306, GLU345, ASN310, ASP271, THR313, GLU387	HIS252, THR254, HIS253, PRO291, ASP326, ILE214, PRO210
Most involved peptide residues	TRP3, LYS2, CYS10, SER1, GLN7, GLN6	TRP3, GLN7, GLN6, PRO21, CYS10, ALA14, LYS24, ARG25
Main specific interactions	H-bonds with TRP390, LYS386, THR313, ASP271; π - π stacking with TRP348	Salt bridge between ASP326 and LYS24; H-bonds with THR254; π - π stacking with HIS253
NLS-region involvement	Not applicable	Present, mainly through PRO21, LYS24, and ARG25



MTT cell viability assay

The effect of the two peptide variants on A549 cell viability was evaluated using the MTT assay. Compound A represented the modified 27-amino-acid peptide containing the rigid linker and nuclear localization signal, whereas Compound B represented the native/core peptide sequence. The purpose of this experiment was to determine whether the NLS-modified peptide showed stronger antiproliferative activity than the native/core peptide in A549 lung adenocarcinoma cells. A549 cells were treated with Compound A or Compound B at concentrations ranging from 10.93 to 700 μM and evaluated after 24, 48, and 72 h. Cell viability was expressed as a percentage of untreated control cells, and IC_{50} values were calculated using nonlinear dose–response curve fitting.

After 24 h, both compounds reduced A549 cell viability, but Compound A produced a stronger response than Compound B. At 10.93 μM , cell viability was 67.07% for Compound A and 74.42% for Compound B. At 87.5 μM , Compound A reduced viability to 14.72%, whereas Compound B-treated cells retained 67.29% viability. At the highest tested concentration, 700 μM , viability decreased to 1.70% with Compound A and 19.32% with Compound B. The calculated IC_{50} values were 52.69 μM for Compound A and 238.26 μM for Compound B, indicating that Compound A was approximately 4.5-fold more potent than Compound B at 24 h. After 48 h, both compounds showed greater reduction in cell viability compared with the 24 h exposure. Compound A reduced viability to 32.11% at 43.75 μM and to 1.49% at 700 μM . Compound B also showed increased activity over time, with viability decreasing to 46.67% at 87.5 μM and 24.81% at 700 μM . The IC_{50} value of Compound A decreased to 37.35 μM , while Compound B showed an IC_{50} of 81.42 μM , indicating that

Compound A remained approximately 2.2-fold more potent at this time point.

After 72 h, the fitted IC_{50} values showed a further time-dependent increase in activity for both compounds. Compound A reached an IC_{50} of 18.60 μM , while Compound B reached an IC_{50} of 43.34 μM . However, the concentration–response profile of Compound A at 72 h was not fully monotonic across all tested concentrations, particularly between the lower and middle dose range. Therefore, the 72 h result was interpreted based on the overall fitted dose–response trend rather than on individual concentration points alone. Despite this variability, Compound A maintained a lower IC_{50} than Compound B at 72 h, remaining approximately 2.3-fold more potent based on the fitted IC_{50} values.

Across all exposure times, Compound A consistently showed lower fitted IC_{50} values than Compound B. The IC_{50} values of Compound A decreased from 52.69 μM at 24 h to 37.35 μM at 48 h and 18.60 μM at 72 h. Compound B followed the same general time-dependent pattern, with IC_{50} values decreasing from 238.26 μM to 81.42 μM and then 43.34 μM . These findings indicate that both compounds reduced A549 cell viability in a time-dependent manner, with the modified NLS-containing peptide showing stronger antiproliferative activity than the native/core peptide.

Overall, the MTT results support the hypothesis that nuclear-targeted modification of the lunasin-derived peptide enhanced its biological activity in A549 cells. However, because the MTT assay measures metabolic activity rather than the direct mechanism of cell death, these results should be interpreted as evidence of reduced cell viability and antiproliferative activity, not as direct proof of AP-1 inhibition, apoptosis, or nuclear uptake. Further mechanistic assays are required to define the exact mode of action.



Table 3. A549 cell viability and IC₅₀ values following treatment with Compound A and Compound B for 24, 48, and 72 h.

Conc. (μ M)	24 hr				48 hr				72 hr			
	A Viab. (%)	A SD	B Viab. (%)	B SD	A Viab. (%)	A SD	B Viab. (%)	B SD	A Viab. (%)	A SD	B Viab. (%)	B SD
Untreated	100.00	0.004	100.00	0.010	100.00	0.003	100.00	0.008	100.00	0.004	100.00	0.001
10.93	67.07	0.004	74.42	0.005	66.90	0.005	74.75	0.004	90.92	0.015	69.95	0.026
21.8	61.26	0.014	72.56	0.010	60.93	0.013	72.46	0.011	49.03	0.015	62.72	0.032
43.75	59.96	0.047	65.53	0.016	32.11	0.005	67.10	0.002	50.89	0.034	42.05	0.036
87.5	14.72	0.001	67.29	0.001	17.30	0.001	46.67	0.047	44.84	0.035	21.30	0.002
175	14.21	0.001	56.20	0.017	16.80	0.000	24.70	0.027	22.42	0.017	13.35	0.013
350	10.11	0.003	19.76	0.004	10.93	0.003	25.46	0.001	17.69	0.019	14.94	0.007
700	1.70	0.003	19.32	0.002	1.49	0.004	24.81	0.002	20.95	0.005	12.88	0.001
IC ₅₀	52.69 μ M		238.26 μ M		37.35 μ M		81.42 μ M		18.60 μ M		43.34 μ M	
R value	0.920		0.917		0.970		0.996		0.882		0.999	

Data are presented as mean cell viability (%) with corresponding SD values obtained from replicate absorbance-based calculations. Cell viability was calculated relative to untreated control cells. IC₅₀ values were calculated using nonlinear dose–response curve fitting. Compound A: NLS-modified 27-amino acid peptide; compound B: native/core peptide.

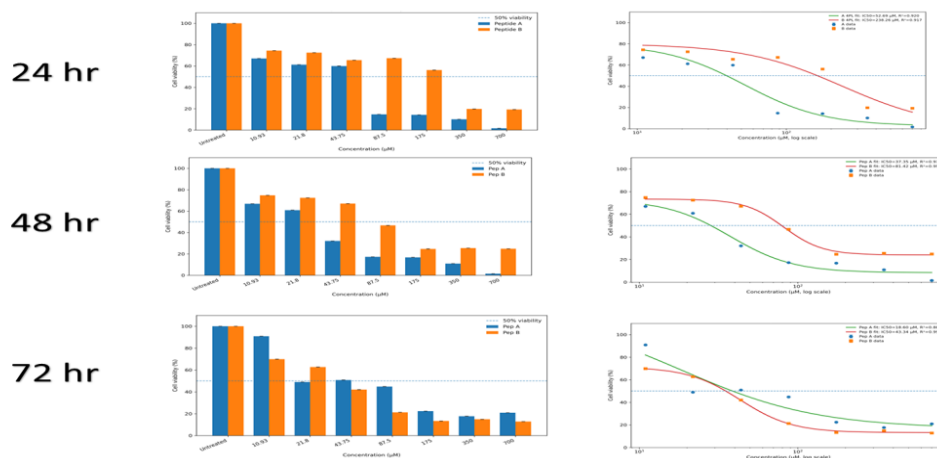


Figure 5. Dose–response effect of Compound A and Compound B on A549 cell viability after 24, 48, and 72 h of treatment. A549 cells were treated with serial concentrations of Compound A or Compound B ranging from 10.93 to 700 μ M. Cell viability was measured using the MTT assay and expressed as a percentage of untreated control cells. Dose–response curves were generated using nonlinear concentration–response curve fitting, and IC₅₀ values were calculated for each compound at each time point. Compound A showed lower fitted IC₅₀ values than Compound B at all tested time points, indicating stronger antiproliferative activity of the modified NLS-containing peptide.

Error bars represent SD from triplicate measurements.



Morphological changes in treated cells

Microscopic examination was performed to provide qualitative visual support for the MTT findings. Untreated A549 cells showed the expected adherent growth pattern, with high cell density and preserved cellular morphology. In contrast, peptide-treated cells displayed visible treatment-associated changes, including reduced cell density, altered cell shape, partial loss of adherence, shrinkage, and detachment.

These changes were more evident in treated groups than in untreated control cells and were generally consistent with the reduction

in cell viability observed in the MTT assay. Cells treated with Compound A showed more pronounced reduction in cell density and morphological alteration, in agreement with its lower IC_{50} values. Compound B also produced visible morphological changes, although these were less consistent with a suppressive transcriptional pattern in the later RT-qPCR analysis. Therefore, the microscopic observations were interpreted as supportive evidence of treatment-associated cellular stress and reduced viability, but not as proof of a specific mechanism of cell death.

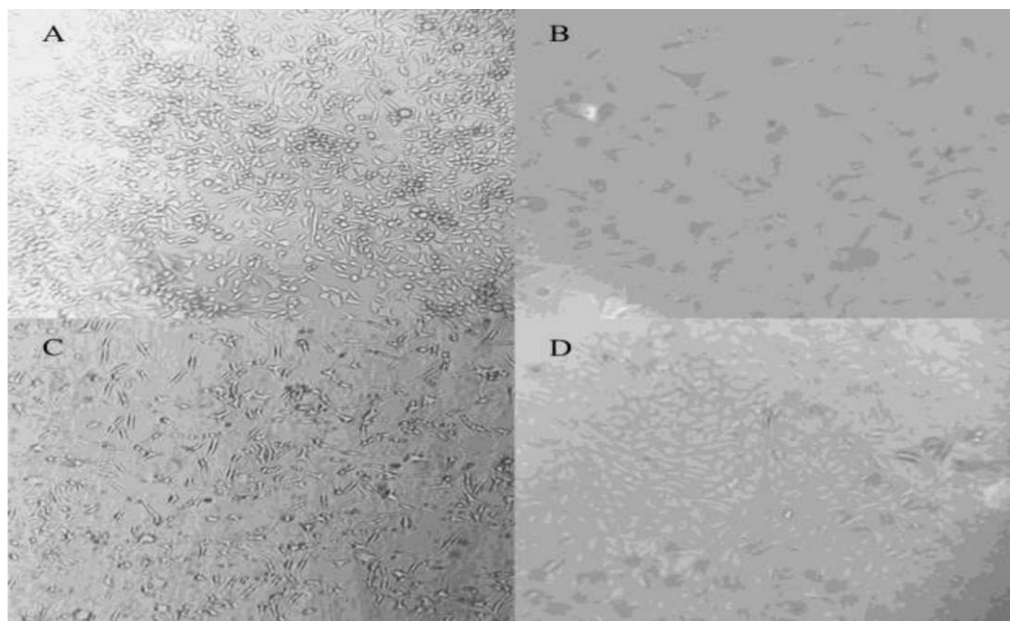


Figure 6. Morphological changes of A549 cells under different treatment conditions. Representative inverted-microscope images showing A549 cell morphology after peptide treatment. (A) Untreated control cells showing preserved adherent morphology and high confluency. (B) Cells treated with Compound A showing reduced cell density and altered morphology. (C) Cells treated with Compound B showing moderate morphological changes with partial loss of normal cellular architecture. (D) Cells treated with a higher concentration of Compound B showing more pronounced treatment-associated morphological changes, including cell shrinkage and reduced adherence. These images provide qualitative support for the MTT findings but do not define the mechanism of cell death.

RT-qPCR analysis

RT-qPCR analysis was performed to examine whether peptide treatment influenced the expression of selected AP-1-associated downstream genes. Based on the bioinformatics and network-guided analysis, MMP9, CCND1, and VEGFA were selected as functional gene-expression markers related to invasion, proliferation, and angiogenesis, respectively. GAPDH was used as the internal reference gene, and relative expression was calculated using the $2^{-\Delta\Delta C_t}$ method.

For MMP9, treatment with Compound A produced a marked reduction in gene expression. The mean fold change decreased to 0.0739, corresponding to an approximately 13.5-fold reduction compared with the untreated control. This reduction was statistically significant, with a p-value of 0.0301. Compound B also reduced MMP9 expression, with a mean fold change of 0.6792; however, this change did not reach statistical significance ($p = 0.5153$).

For CCND1, Compound A reduced gene expression to a mean fold change of 0.1024, indicating a clear downward trend compared with the untreated control. This corresponds to an approximately 9.8-fold reduction. However, this reduction was not statistically significant ($p = 0.0937$) and should therefore

be described as a strong non-significant decreasing trend rather than confirmed suppression. Compound B showed a mean fold change of 2.0706, but the result was variable and did not reach statistical significance ($p = 0.5879$).

For VEGFA, Compound A produced a clear and statistically significant reduction in expression. The mean fold change decreased to 0.1676, corresponding to an approximately 6-fold reduction compared with the untreated control, with a p-value of 0.0072. In contrast, Compound B showed a mean fold change of 1.4035, but this change was not statistically significant ($p = 0.7161$).

Overall, the RT-qPCR results showed that Compound A reduced the expression of all three tested genes, with statistically significant reductions in MMP9 and VEGFA and a strong non-significant decreasing trend in CCND1. In contrast, Compound B did not produce statistically significant suppression of the tested genes. These findings support the stronger biological effect of the optimized NLS-containing peptide compared with the native/core peptide in A549 cells. However, because RT-qPCR was performed using only two independent biological replicates, these results should be interpreted as preliminary gene-expression evidence rather than definitive mechanistic proof.

Table 4. Relative expression of AP-1-associated genes in A549 cells after treatment with Compound A and Compound B.

Gene	Compound A mean fold change	P-value	Compound B mean fold change	P-value	Interpretation
MMP9	0.0739	0.0301	0.6792	0.5153	Compound A significantly reduced MMP9 expression; Compound B showed a non-significant reduction.
CCND1	0.1024	0.0937	2.0706	0.5879	Compound A showed a strong decreasing trend in CCND1 expression, but this was not statistically significant; Compound B showed a non-significant increase.
VEGFA	0.1676	0.0072	1.4035	0.7161	Compound A significantly reduced VEGFA expression; Compound B showed a non-significant increase.



*Gene expression was normalized to GAPDH and calculated using the $2^{-\Delta\Delta C_t}$ method relative to untreated control cells. Data are presented as mean fold change from two independent biological replicates. P-values were calculated using the statistical test described in the Methods section and should be interpreted cautiously because of the limited replicate number. Compound A: NLS-modified 27-amino-acid peptide; Compound B: native/core peptide.

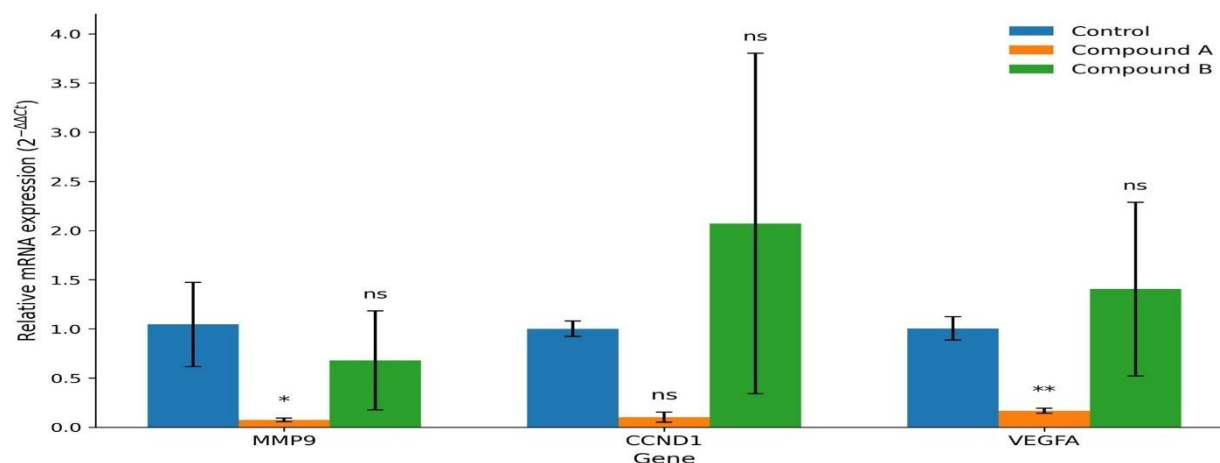


Figure 7. Relative mRNA expression of MMP9, CCND1, and VEGFA in A549 cells after treatment with Compound A and Compound B. Gene expression was normalized to GAPDH and calculated using the $2^{-\Delta\Delta C_t}$ method relative to untreated control cells. Bars represent mean fold change, and error bars represent SEM from two independent biological replicates. Dots represent individual biological replicates. Compound A significantly reduced MMP9 and VEGFA expression and showed a strong but non-significant decreasing trend for CCND1. Compound B did not produce statistically significant suppression of the tested genes. * $p < 0.05$, ** $p < 0.01$ versus untreated control; ns, not significant.

Discussion

The present study evaluated whether nuclear-targeted engineering of a previously designed lunasin-derived peptide could improve its biological activity in A549 lung adenocarcinoma cells. This work represents an early experimental validation step following computational peptide design. The study integrated network-guided gene selection, importin- α docking, MTT-based cell viability assessment, morphological examination, and RT-qPCR analysis of selected AP-1-associated downstream genes. This combined workflow allowed a comparative evaluation of the modified NLS-containing peptide, Compound A, and the

native/core peptide, Compound B, while avoiding reliance on a single computational or biological readout. Such an integrated approach is consistent with the growing use of network-based and structure-guided strategies to support rational candidate prioritization in drug discovery (23).

AP-1 was selected as the central transcriptional framework because of its established involvement in cancer-related gene regulation. AP-1 integrates signals from multiple upstream pathways and regulates genes involved in proliferation, survival, invasion, inflammation, angiogenesis, and cellular adaptation (24,25). However, the bioinformatics findings in the present study should be interpreted carefully. The GEPIA2



survival analysis did not show statistically significant overall survival separation for individual FOS or JUN expression alone. Therefore, AP-1 was not considered validated solely on the basis of single-gene survival association. Instead, its selection was supported by combined evidence from altered expression patterns, dense STRING/Cytoscape network connectivity, functional enrichment, and published literature describing AP-1 as a context-dependent transcriptional regulator in cancer biology (24,25).

The difficulty of targeting AP-1 is related to the broader challenge of modulating transcription factors. Unlike enzymes or receptors with well-defined ligand-binding pockets, transcription factors often act through extended protein–protein or protein–DNA interfaces, which limits the effectiveness of many conventional small molecules (6). Peptide-based approaches may offer an alternative because peptides can provide larger and more flexible interaction surfaces capable of engaging shallow or extended binding regions (7). In this study, the peptide modification strategy was based on adding a rigid EAAAK linker and a classical nuclear localization signal to the selected lunasin-derived core sequence. The purpose of this design was to preserve the AP-1-interacting core while improving compatibility with nuclear-directed delivery. Rigid linkers such as EAAAK are commonly used to separate functional domains and reduce unfavorable interference between them (26).

The importin- α docking analysis provided supportive structural information for this design. Compound A showed broader receptor engagement than Compound B and involved residues located within or near the NLS-containing region, including PRO21, LYS24, and ARG25. The predicted salt bridge between peptide LYS24 and importin- α ASP326 suggested that the appended basic

region may contribute to importin- α recognition. This agrees with the known role of lysine- and arginine-rich motifs in classical NLS recognition (9,10). Nevertheless, these docking results should be interpreted cautiously. Docking does not prove cellular uptake or nuclear transport, and the reported van der Waals clashes, particularly for Compound A, indicate that the predicted pose may require further refinement. Therefore, the importin- α docking result supports the structural plausibility of the NLS design but does not experimentally confirm nuclear accumulation.

The MTT results supported the biological advantage of the modified peptide. Both compounds reduced A549 cell viability, indicating that the native/core sequence itself possesses measurable biological activity. However, Compound A was consistently more potent than Compound B at all tested time points. The IC₅₀ values of Compound A decreased from 52.69 μ M at 24 h to 37.35 μ M at 48 h and 18.60 μ M at 72 h. In comparison, Compound B showed higher IC₅₀ values of 238.26, 81.42, and 43.34 μ M at the same time points. This pattern indicates that the NLS-modified peptide produced a stronger time-dependent reduction in A549 cell viability than the native/core peptide.

The time-dependent decrease in IC₅₀ values is compatible with a progressive biological effect; however, it should not be interpreted as proof of a specific mechanism. The MTT assay measures metabolic activity and indirectly estimates viable cells, but it does not distinguish between apoptosis, necrosis, cell-cycle arrest, metabolic suppression, or reduced proliferation (27). Therefore, the MTT results are best described as evidence of reduced cell viability and antiproliferative activity rather than direct proof of AP-1 inhibition or a defined mode of cell death. Additional mechanistic assays would be required to clarify whether the observed



response is due to apoptosis, cell-cycle arrest, or other forms of treatment-associated cellular stress.

The morphological observations were consistent with the MTT findings. Treated A549 cells showed reduced cell density and visible treatment-associated changes, including altered cell shape, shrinkage, and partial loss of adherence compared with untreated control cells. These observations visually support the reduction in cell viability observed after peptide treatment. However, morphology alone cannot define the mechanism of cell death. Future assays such as Annexin V/PI staining, caspase activity measurement, mitochondrial membrane-potential analysis, and cell-cycle profiling would be required to determine the biological process responsible for the observed reduction in viability.

RT-qPCR analysis provided molecular support for the stronger activity of Compound A. Among the tested genes, MMP9 showed the clearest reduction after Compound A treatment, with a mean fold change of 0.0739, corresponding to approximately a 13.5-fold decrease relative to untreated control. MMP9 is strongly associated with extracellular matrix degradation, tumor invasion, and metastatic behavior (28). Because AP-1 has been implicated in the regulation of invasion- and matrix-remodeling-related genes, the reduction in MMP9 supports the possibility that Compound A influences AP-1-associated invasive transcriptional outputs (25,28). However, this result should be interpreted as downstream gene-expression evidence rather than direct proof of AP-1 inhibition.

VEGFA was also reduced after Compound A treatment, with a mean fold change of 0.1676, corresponding to approximately a 6-fold decrease compared with untreated control. VEGFA is a major regulator of angiogenesis and tumor-supportive vascular

signaling (29). The reduction in VEGFA therefore suggests that Compound A may affect angiogenesis-associated transcriptional responses. However, VEGFA expression is regulated by multiple transcriptional pathways, including HIF-1 α , SP1, STAT3, and AP-1 (30). Therefore, the VEGFA result should be interpreted as evidence of altered angiogenesis-related gene expression, not as proof that AP-1 alone mediated the observed response.

The CCND1 result also supported a possible antiproliferative transcriptional effect but required more cautious interpretation. Compound A reduced CCND1 expression to a mean fold change of 0.1024, representing a strong downward trend. Because CCND1 encodes cyclin D1, a key regulator of cell-cycle progression, this trend is consistent with the reduced viability observed in the MTT assay (31). However, the change did not reach conventional statistical significance. Therefore, CCND1 should be described as a preliminary decreasing trend rather than confirmed suppression. This finding requires validation using additional biological replicates and protein-level assessment of cyclin D1.

An important point raised by the gene-expression results was the different behavior of Compound B. Although Compound B reduced A549 cell viability in the MTT assay, it did not produce a consistent suppressive transcriptional pattern for the selected genes. It showed a non-significant numerical reduction in MMP9, but non-significant increases in CCND1 and VEGFA. This apparent discrepancy may reflect the fact that MTT measures metabolic viability rather than a specific transcriptional mechanism. Therefore, the reduced viability observed with Compound B may involve AP-1-independent effects, delayed cellular stress, altered metabolism, or other mechanisms not captured by the selected 24 h RT-qPCR readouts. The non-significant increases in



CCND1 and VEGFA may also represent variable transcriptional responses among the remaining viable cells rather than a confirmed pro-tumorigenic effect. Because these changes were not statistically significant and were based on only two biological replicates, no firm mechanistic conclusion can be drawn for Compound B.

Together, the MTT and RT-qPCR results indicate that Compound A produced stronger phenotypic and gene-expression effects than Compound B. Compound A showed lower IC₅₀ values at all time points and reduced the expression of MMP9 and VEGFA, with a marked decreasing trend for CCND1. This agreement between reduced viability and altered expression of cancer-associated genes supports the biological activity of the modified peptide. Nevertheless, these findings should be considered preliminary validation rather than definitive mechanistic proof. Direct confirmation of AP-1 inhibition would require additional assays such as AP-1 luciferase reporter analysis, assessment of AP-1 DNA-binding activity, chromatin immunoprecipitation for AP-1 occupancy at target promoters, or Western blotting of AP-1-related downstream proteins.

A strength of this study is that it connects computational peptide engineering with early in vitro validation. The computational workflow provided a rationale for peptide selection and modification, while the experimental work tested whether the modified construct produced measurable biological effects in A549 cells. This integrated approach is useful because docking results alone cannot establish biological activity, while cell-based assays without a mechanistic rationale may remain descriptive. By combining network analysis, importin- α docking, MTT assay, morphology, and RT-qPCR, the study provides a structured early validation framework for the NLS-modified peptide.

Several limitations must be acknowledged. First, RT-qPCR was performed using only two biological replicates, which limits statistical power and prevents definitive interpretation of some gene-expression changes, particularly CCND1. This limitation is important because qPCR reliability depends strongly on adequate replication, transparent reporting, and cautious interpretation of fold-change values (32). Second, mRNA expression does not necessarily reflect protein abundance or functional protein activity. Western blotting or ELISA would be needed to confirm whether the observed transcriptional changes are reflected at the protein level. For MMP9, gelatin zymography would be particularly useful to determine whether reduced mRNA expression corresponds to reduced enzymatic activity. Third, the study was performed only in A549 cells, so the findings cannot yet be generalized to other NSCLC models. Testing additional lung cancer cell lines, such as H1299 or H460, and a non-malignant lung epithelial cell line such as BEAS-2B, would help evaluate selectivity and therapeutic window.

The use of only two independent biological replicates for RT-qPCR is a limitation of the present study and reduces the statistical power of the gene-expression analysis. Therefore, the qPCR findings were interpreted as preliminary and hypothesis-supporting rather than definitive mechanistic validation.

Another important limitation is that nuclear delivery was inferred indirectly from importin- α docking and biological response, but direct nuclear localization was not confirmed in this study. Future experiments should include confocal microscopy with nuclear staining, quantitative nuclear/cytoplasmic fluorescence analysis, and/or nuclear-cytoplasmic fractionation. These experiments would clarify whether Compound A accumulates in the nucleus



more effectively than Compound B. In addition, apoptosis assays, cell-cycle analysis, AP-1 reporter assays, and protein-level validation would be required to define the mechanism more precisely.

Despite these limitations, the current findings support Compound A as a biologically active modified peptide candidate. Compared with the native/core peptide, Compound A showed stronger reduction of A549 cell viability and more coherent modulation of selected AP-1-associated downstream genes, particularly MMP9 and VEGFA. These results suggest that nuclear-targeted modification enhanced the biological activity of the lunasin-derived peptide in this experimental model. However, the data do not yet prove direct AP-1 inhibition or confirmed nuclear import. The most appropriate interpretation is that Compound A produced antiproliferative activity and altered selected AP-1-associated gene-expression outputs in A549 cells, supporting its further mechanistic evaluation and optimization.

Conclusion

This study evaluated the early in vitro biological activity of a rationally engineered lunasin-derived peptide in A549 lung adenocarcinoma cells. The work extended previous computational peptide-prioritization efforts toward experimental validation by comparing a modified NLS-containing peptide, Compound A, with the native/core peptide, Compound B.

The MTT assay showed that both peptides reduced A549 cell viability in a time-dependent manner; however, Compound A consistently demonstrated stronger antiproliferative activity across 24, 48, and 72 h. This improved activity was consistent with the nuclear-targeted design strategy, in which the AP-1-interacting core sequence was combined with a rigid EAAAK linker and a nuclear localization signal. Importin- α

docking further supported the structural plausibility of this design by showing broader receptor engagement and direct involvement of NLS-region residues in Compound A.

At the gene-expression level, Compound A reduced the expression of all three tested AP-1-associated genes, with significant reductions in MMP9 and VEGFA and a marked non-significant decreasing trend in CCND1. In contrast, Compound B did not produce statistically significant suppression of the tested genes under the same experimental conditions. These findings suggest that the modified peptide may influence cancer-related transcriptional outputs associated with invasion, angiogenesis, and proliferation.

Overall, Compound A showed stronger antiproliferative activity and a more coherent AP-1-associated gene-expression response than the native/core peptide in A549 cells. However, the current findings remain preliminary and do not yet prove direct AP-1 inhibition or confirmed nuclear transport. Further validation using AP-1 reporter assays, nuclear localization studies, protein-level analysis, apoptosis and cell-cycle assays, and additional cancer and non-malignant cell models is required.

In conclusion, the modified 27-amino-acid NLS-containing lunasin-derived peptide represents a promising candidate for further mechanistic investigation as a potential AP-1-associated anticancer peptide.

References

1. Bray F, Laversanne M, Sung H, Ferlay J, Siegel RL, Soerjomataram I, et al. Global cancer statistics 2022: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin.* 2024;74(3):229–263.
2. Herbst RS, Morgensztern D, Boshoff C. The biology and management of non-small cell lung cancer. *Nature.* 2018;553(7689):446–454.



3. Papavassiliou KA, Anagnostopoulos N, Papavassiliou AG. Lung cancer through transcription factors. *Int J Mol Sci.* 2023;24(11):9461.
4. Angel P, Karin M. The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. *Biochim Biophys Acta.* 1991;1072(2–3):129–157.
5. Shaulian E, Karin M. AP-1 as a regulator of cell life and death. *Nat Cell Biol.* 2002;4(5):E131–E136.
6. Darnell JE Jr. Transcription factors as targets for cancer therapy. *Nat Rev Cancer.* 2002;2(10):740–749.
7. Craik DJ, Fairlie DP, Liras S, Price D. The future of peptide-based drugs. *Chem Biol Drug Des.* 2013;81(1):136–147.
8. Al-Juboori ZA, Arif IS, Albanna A. In silico engineering of nuclear-targeted peptides targeting the AP-1 transcriptional complex. *Pharmacia.* 2026;73:e190360. doi:10.3897/pharmacia.73.e190360.
9. Kalderon D, Richardson WD, Markham AF, Smith AE. Sequence requirements for nuclear location of simian virus 40 large-T antigen. *Nature.* 1984;311(5981):33–38.
10. Lange A, Mills RE, Lange CJ, Stewart M, Devine SE, Corbett AH. Classical nuclear localization signals: definition, function, and interaction with importin α . *J Biol Chem.* 2007;282(8):5101–5105.
11. Szklarczyk D, Kirsch R, Koutrouli M, Nastou K, Mehryary F, Hachilif R, et al. The STRING database in 2023: protein–protein association networks and functional enrichment analyses for any sequenced genome of interest. *Nucleic Acids Res.* 2023;51(D1):D638–D646.
12. Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, et al. Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res.* 2003;13(11):2498–2504.
13. Berman HM, Westbrook J, Feng Z, Gilliland G, Bhat TN, Weissig H, et al. The Protein Data Bank. *Nucleic Acids Res.* 2000;28(1):235–242.
14. Schrödinger Release 2025-1: Protein Preparation Wizard; Epik, Schrödinger, LLC, New York, NY, 2025; Impact, Schrödinger, LLC, New York, NY, 2025; Prime, Schrödinger, LLC, New York, NY, 2025.
15. Lu C, Wu C, Ghoreishi D, Chen W, Wang L, Damm W, et al. OPLS4: improving force field accuracy on challenging regimes of chemical space. *J Chem Theory Comput.* 2021;17(7):4291–4300.
16. Kozakov D, Brenke R, Comeau SR, Vajda S. PIPER: an FFT-based protein docking program with pairwise potentials. *Proteins.* 2006;65(2):392–406.
17. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods.* 1983;65(1–2):55–63.
18. Sylvester PW. Optimization of the tetrazolium dye (MTT) colorimetric assay for cellular growth and viability. *Methods Mol Biol.* 2011;716:157–168.
19. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Anal Biochem.* 1987;162(1):156–159.
20. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. *Methods.* 2001;25(4):402–408.
21. Al-Khfajy WS. The effect of farnesoid X receptor agonist (INT 747) and fasting mimicking diet on metastatic castration-resistant prostate cancer cell line (PC-3) [dissertation]. Baghdad: College of Pharmacy, Mustansiriyah University; 2022.
22. Sarkar C, Chakroborty D, Goswami S, Fan H, Mo X, Basu S. VEGF-A controls the expression of its regulator of angiogenic functions, dopamine D2 receptor, on endothelial cells. *J Cell Sci.*



- 2022;135(11):jcs259617.
doi:10.1242/jcs.259617.
23. Csermely P, Korcsmáros T, Kiss HJM, London G, Nussinov R. Structure and dynamics of molecular networks: a novel paradigm of drug discovery: a comprehensive review. *Pharmacol Ther.* 2013;138(3):333–408.
24. Eferl R, Wagner EF. AP-1: a double-edged sword in tumorigenesis. *Nat Rev Cancer.* 2003;3(11):859–868.
25. Ozanne BW, Spence HJ, McGarry LC, Hennigan RF. Transcription factors control invasion: AP-1 the first among equals. *Oncogene.* 2007;26(1):1–10.
26. Chen X, Zaro JL, Shen WC. Fusion protein linkers: property, design and functionality. *Adv Drug Deliv Rev.* 2013;65(10):1357–1369.
27. Adan A, Kiraz Y, Baran Y. Cell proliferation and cytotoxicity assays. *Curr Pharm Biotechnol.* 2016;17(14):1213–1221.
28. Yan C, Boyd DD. Regulation of matrix metalloproteinase gene expression. *J Cell Physiol.* 2007;211(1):19–26.
29. Ferrara N. Vascular endothelial growth factor: basic science and clinical progress. *Endocr Rev.* 2004;25(4):581–611.
30. Pages G, Pouyssegur J. Transcriptional regulation of the vascular endothelial growth factor gene: a concert of activating factors. *Cardiovasc Res.* 2005;65(3):564–573.
31. Musgrove EA, Caldon CE, Barraclough J, Stone A, Sutherland RL. Cyclin D as a therapeutic target in cancer. *Nat Rev Cancer.* 2011;11(8):558–572.
32. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem.* 2009;55(4):611

