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Original Article

Unveiling Betulinic Acid as a Potent CDK4 Inhibitor for Cancer Therapeutics

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ABSTRACT

Background: CDK4 play a pivotal role in cell cycle regulation, making it a critical players in the development and progression of cancer. In recent years, there has been a growing interest in targeting CDK4 for cancer therapeutics, with a focus on the identification and development of small molecule inhibitors. **Materials and Methods:** This work, using a strong *in silico* and *in vitro* methodology, reveals Betulinic Acid's inhibitory efficacy against CDK4 for cancer therapy. Betulinic Acid, a pentacyclic triterpenoid with a variety of characteristics, has emerged as a promising CDK4 inhibitor. **Results:** The study identifies key CDK4 binding sites using high-resolution structural modeling and cavity detection. Betulinic Acid's highest fitness and predicted binding affinity were found, supporting its drug-likeness properties. Its pharmacokinetic viability, biological properties, and cytotoxicity assays show its concentration-dependent effects on cancer cells (A549 and NCI-H460). Molecular-level validations reveal a significant decrease in CDK4 mRNA expression and kinase activity, reinforcing its potential as a CDK4 inhibitor. **Conclusion:** In conclusion, this comprehensive study bridges structural insights with experimental validations, positioning Betulinic Acid as a promising therapeutic agent for CDK4 inhibition in cancer, particularly lung cancer. The findings contribute significantly to drug discovery, paving the way for further preclinical and clinical investigations in the quest for effective cancer treatments.

Keywords: Betulinic Acid, Cancer therapeutics, CDK4 inhibition, Lung cancer treatment, Molecular docking, Pharmacokinetic viability.

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INTRODUCTION

Cancer is a complex group of diseases characterized by the uncontrolled growth and spread of abnormal cells.[1,2] It can affect virtually any tissue or organ in the body, disrupting normal cellular functions. The causes of cancer are diverse, involving genetic mutations, environmental factors, and lifestyle choices.[3] Cancer manifests in various forms, each with distinct symptoms and treatment approaches.[4-6] Early detection and advancements in medical research have improved prognosis, but cancer remains a global health challenge. Treatment modalities include surgery, chemotherapy, radiation therapy, immunotherapy, and targeted therapies.[7,8] Ongoing research and awareness efforts aim to enhance prevention, diagnosis, and therapeutic strategies against this formidable disease.

Cyclin-Dependent Kinases (CDKs) are pivotal regulators of the cell cycle, orchestrating the intricate process of cell division. These enzymes collaborate with cyclins, forming complexes that act as molecular switches, driving cells through distinct phases such as G1, S, G2, and M.[9,10] CDKs phosphorylate key substrates, activating or inhibiting specific pathways to ensure orderly progression.[11] Their finely tuned activity governs crucial checkpoints, ensuring DNA replication fidelity and proper cell division. Dysregulation of CDKs is implicated in various diseases, including cancer, emphasizing their significance in maintaining cellular homeostasis.[12]

CDK4 play a pivotal role in cell cycle regulation, making it a critical players in the development and progression of cancer.[13,14] This

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serine/threonine kinase, when aberrantly activated, contribute to uncontrolled cell proliferation, a hallmark of cancer.[12] In recent years, there has been a growing interest in targeting CDK4 for cancer therapeutics, with a focus on the identification and development of small molecule inhibitors.

CDK4 is a member of the cyclin-dependent kinase family, which are key regulators of cell cycle progression.[15] Their activity is tightly controlled by binding to cyclins, specifically cyclin D1 in the case of CDK4 and CDK6.[12] The activation of CDK4 and CDK6 triggers the phosphorylation of Retinoblastoma protein (Rb), leading to the release of E2F transcription factors and subsequent progression from the G1 to the S phase of the cell cycle.[16] This transition is a crucial checkpoint, ensuring that the cell has the necessary resources and conditions to commit to cell division.

In many cancer types, the regulation of CDK4 is disrupted, resulting in its overexpression and hyperactivation.[17] This dysregulation leads to unchecked cell cycle progression and uncontrolled cellular proliferation, contributing to tumor development and growth.[18-20] Overexpression of cyclin D1, the partner cyclin for CDK4, is frequently observed in various cancers, further emphasizing the significance of this kinase in oncogenesis.[21]

The clinical significance of CDK4 in cancer is underscored by its association with poor prognosis and aggressive tumor behaviour.[22,23] Studies have demonstrated that elevated CDK4 expression is correlated with advanced stages of cancer, increased tumor size, and decreased overall survival in various malignancies, including breast cancer, melanoma, and glioblastoma.[24-26] This highlights the potential utility of targeting CDK4 as a therapeutic strategy to impede cancer progression.

The recognition of CDK4 as promising therapeutic targets has led to the development of small molecule inhibitors designed to specifically block their kinase activity.[13] These inhibitors work by competitively binding to the ATP-binding pocket of CDK4, preventing its interaction with cyclin D1 and subsequent phosphorylation of Rb.[16,27] Several CDK4/6 inhibitors have been developed and tested in preclinical and clinical settings. Palbociclib, Ribociclib, and Abemaciclib are three FDA-approved CDK4/6 inhibitors that have demonstrated efficacy in various cancers.[28-30] Palbociclib, for instance, has shown significant clinical benefit in combination with endocrine therapy for Estrogen Receptor-positive (ER^+) breast cancer.[31] Ribociclib and Abemaciclib have also demonstrated efficacy in breast cancer and have expanded into other malignancies, such as advanced melanoma.[29] Importantly, the use of CDK4/6 inhibitors in combination with endocrine therapy has shown promise in hormone receptor-positive breast cancer, providing a novel therapeutic strategy for this subtype.[32] Beyond breast cancer, CDK4/6 inhibitors are being explored in other solid tumors and hematologic malignancies.[33]

While CDK4 inhibitors have shown remarkable success in certain cancer types, challenges remain. Resistance to CDK4 inhibitors can develop, necessitating further research to understand the underlying mechanisms and develop new treatment strategies to overcome resistance. The development of CDK4 inhibitors represents a paradigm shift in cancer treatment, providing targeted therapies with favourable safety profiles. Future directions in the development of CDK4 inhibitors include the exploration of novel compounds with improved pharmacokinetic profiles, enhanced selectivity, and reduced side effects. The continued exploration of CDK4 as therapeutic targets, coupled with the refinement of small molecule inhibitors, holds great promise for the future of cancer treatment. This study aims to identify penitential inhibitor small molecules of CDK4 using *in silico* and *in vitro* methods for the therapeutics of cancer. Exploring novel CDK4 inhibitors with improved profiles ensures a continued paradigm shift in cancer therapeutics.

MATERIALS AND METHODS

3D-Structure modelling and preparation of CDK4 receptor

Homology modelling, a widely employed method for predicting the three-dimensional structure of a protein based on similar proteins with known structures, is commonly performed using SWISS-MODEL, a popular web-based tool (https://swissmodel.expasy.org/).[34-36] We used SWISS-MODEL to model the 3D structure of CDK4 by obtaining its FASTA sequence from PubMed and selecting an appropriate template structure (CDK4: PDB ID - 3G33) based on sequence similarity, quality of structure, and biological relevance.[34-37] The resulting high-resolution CDK4 structure was retrieved from the Swiss-MODEL server (Figure 1a) and then visualized using PyMOL and Chimera for structural analysis. To prepare the receptor for molecular docking, we followed standard protocol to refine the CDK4 structures including inspection for steric clashes or missing atoms.[38,39] Subsequently, partial charges were assigned to facilitate molecular docking compatibility before saving in PDBQT format.

Selection and preparation of ligands for molecular docking with CDK4

A collection of ligand molecules was compiled from DrugBank (https://go.drugbank.com/), comprising 1935 approved small molecule entries. The SDF format files of the ligands were acquired and their structures underwent inspection and correction of bond lengths, angles, and torsional angles as necessary. Subsequently, the structures were converted to PDBQT format to enable molecular docking. All the small molecules were subjected to docking against the target molecule CDK4.

Cavity detection on CDK4

Cavity identification within the CDK4 structure was performed using CB-Dock2 (http://cadd.labshare.cn/cb-dock2/), a protein-ligand docking tool. The protein was prepared in a PDB file and uploaded to the CB-Dock2 Server. Different parameters were set based on the characteristics of the target protein, such as probe radius and cavity size. The tool autonomously identified binding sites and cavities within the target protein, calculated their dimensions and locations, and dynamically adjusted the docking box size for optimal coverage during molecular docking. Furthermore, it allowed visualization of detected cavities to aid in further analysis of binding sites.

Molecular docking of Betulinic Acid and CDK4

Molecular docking calculations of predicted top hit against the target proteins were carried out using DockingServer. Gasteiger partial charges were added to the ligand atoms. Non-polar hydrogen atoms were merged, and rotatable bonds were defined. Docking calculations were carried out on CDK4-Betulinic Acid model. Essential hydrogen atoms, Kollman united atom type charges, and solvation parameters were added with the aid of AutoDock tools.[40] Affinity (grid) maps of 21×21×21 Å (x, y, and z) grid points and 0.375 Å spacing were generated using the Autogrid program. AutoDock parameter set- and distance-dependent dielectric functions were used in the calculation of the van der Waals and the electrostatic terms, respectively. Docking simulations were performed using the Lamarckian Genetic Algorithm (LGA) and the Solis and Wets local search method.[41] Initial position, orientation, and torsions of the ligand molecules were set randomly. All rotatable torsions were released during docking. Each docking experiment was derived from 2 different runs that were set to terminate after a maximum of 250000 energy evaluations. The population size was set to 150. During the search, a translational step of 0.2 Å, and quaternion and torsion steps of 5 were applied.

Additionally, the protein-ligand complex was redocked using CB-Dock2 to validate the binding affinity of protein-ligand complex.

Assessment of ADMET properties of Betulinic Acid

ADMET properties were assessed using pkCSM (Pharmacokinetics and Chemistry of Small Molecules) online tool (https://biosig.unimelb.edu.au/pkcsm/). This tool predicts pharmacokinetic and toxicity properties of small molecules, including ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) properties. The lead molecule Betulinic Acid in SMILES format was uploaded to pkCSM online tool to determine the ADMET properties.

Prediction of Activity Spectra for Betulinic Acid using PASS online and Swiss Target Prediction tool

In the realm of chemoinformatics and drug discovery, the prediction of activity spectra for ligand is an important step. Current study employed PASS online (http://www.pharmaexpert.ru/passonline/) and Swiss Target Prediction tool (http://www.swisstargetprediction.ch/) to determine the biological activity spectrum associated with the Betulinic Acid. PASS (Prediction of Activity Spectra for Substances) Online is a web-based platform that provides predictions for the biological and pharmacological activities of chemical compounds. It is a tool designed to assist researchers in identifying potential activities and properties of small molecules. The predictions generated by PASS are based on the analysis of chemical structures and their relationships to known activities from a vast database. SwissTargetPrediction is a web-based tool developed to predict potential targets for small molecules or drugs. It is designed to facilitate the identification of proteins that may interact with a given compound. This information can be valuable in drug discovery and understanding the potential effects of a compound in biological systems.

The SMILES notation was uploaded to PASS online and SwissTargetPrediction tool to determine the biological activity spectrum of Betulinic Acid.

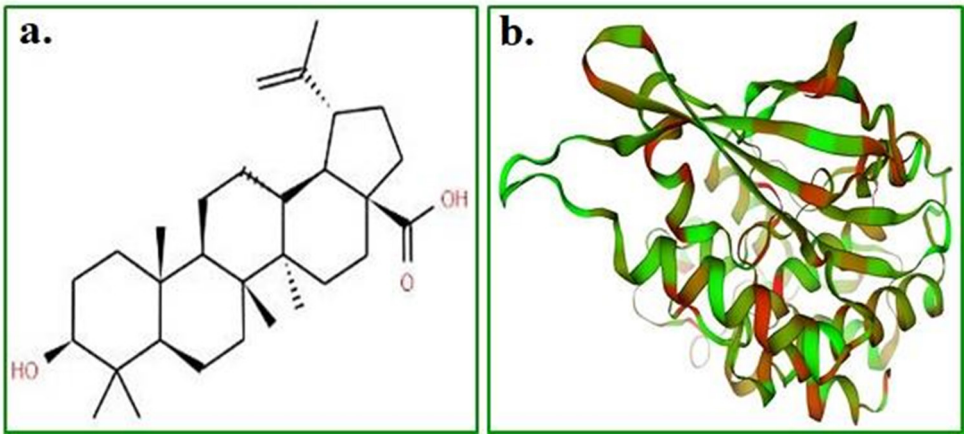


Figure 1: (a) Structure of Betulenic acid; (b) 3D structure of CDK4.

Test compound and MRC-9, A549, and NCI-H460 cell lines

Betulinic Acid (>97.0%(GC)(T)) was acquired from TCI (CAS RN: 472-15-1) and dissolved in DMSO (Sigma, USA) to create a stock solution at 500ug/mL concentration. MRC-9 cells were cultured and maintained in DMEM, A549 in Ham's F-12K, and NCI-H460 in RPMI medium supplemented with 10% FBS and 1% antibiotics.

Cell culture and cytotoxicity assay

Initially, MRC-9 cells were cultivated in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. Cells were maintained at 37°C in a humidified incubator with 5% CO2 to reach the confluency. Thereafter, MRC-9 cells were seeded into 96-well plates at a desired density (e.g., 5,000 cells/well) and allow to adhere and grow for 24 hr. Cells were treated with experimental compound Betulenic acid (Conc: 0 to 500ug) for 72 hr. Control wells were included without treatment. 5 mg/mL MTT solution in PBS was prepared. Culture media was removed from the wells and 100 µL of the MTT solution was added to each well. Plates were incubated at 37°C for 3-4 hr to allow MTT to be converted into formazan crystals by viable cells. After that MTT solution was carefully removed and 100 µL of DMSO was added to each well. Mixture was gently mixed to solubilize the formazan crystals. Absorbance was measured at 570 nm using a microplate reader. Reference wavelength was measured at 630 nm to correct for background. Percentage of cell viability was calculated using the following formula:

$$\text{Cell viability (\%)} = [\text{Absorbance of treated cells} / \text{Absorbance of control cells}] \times 100$$

Additionally, a time-dependent cell viability assay (24, 48, and 72 hr) was conducted to assess the impact of IC_{50} concentration of Betulinic Acid on MRC-9 cells.

mRNA expression and activity of CDK4 in Betulinic Acid treated A549 and NCI-H460 lung cancer cells

A549 and NCI-H460 lung cancer cells were treated with the IC_{50} concentration of Betulinic Acid for 48 hr. RNA was extracted using TRIzol Reagent (Thermo Fisher Scientific). cDNA was synthesized by using cDNA Synthesis Kit (Takara Bio). The quantitative qRT-PCR was conducted using SYBR Green (G-Biosciences) on an Applied Biosystems RT-PCR System. The $2^{-\Delta\Delta CT}$ method was employed to calculate the relative fold change in gene expression. The primer sequences used for the amplification was as: CDK4 5'- CATGTAGACCAGGACCTAAGG-3' (sense) and 5'- AACTGGCGCATCAGATCCTAG-3' (antisense) and GAPDH 5'- AACGTGTCAGTGGTGGACCTG-3' (sense) and 5'-AGTGGGTGTCGCTGTTGAAGT -3' (antisense) [An *et al.*, 1999].

Enzyme inhibition assay

We investigated the impact of Betulinic Acid on CDK4 kinase activity. Briefly, various Betulinic Acid concentrations (0-100 µM) were introduced to CDK6 (2 µM) in a 96-well plate, followed by a 1 hr incubation at 25°C. A reaction mixture with ATP (100 µM) and MgCl2 (10 mM) was added, incubated for 30 min, and halted with BIOMOL® reagent. The green-coloured complex formed was analysed at 620 nm using a plate reader. Malachite green reagent detected inorganic phosphate released during ATP hydrolysis. Native CDK6 activity served as a baseline, marking CDK6 activity without Betulinic Acid as 100%.

Statistical analysis

SPSS was used to evaluate the data. The data is presented as mean ± SD/SEM, pooled from biological triplicate. Mean values of two groups were compared using *t*-test, while the mean values of multiple groups were compared by employing ANOVA. The $p<0.05$ reflects significant differences.

RESULTS

Homology modelling and preparation of CDK4

Figure 1a illustrates the high-resolution structure of the CDK4 model obtained from Swiss-MODEL. The model exhibits a Sequence Identity and coverage of 100%. Notably, the quality of the model is deemed excellent, as indicated by a GMQE score of 0.83 and a QMEANDisCo Global score of 0.79 ± 0.05. Both GMQE and QMEANDisCo global scores provide an overall assessment of model quality, ranging between 0 and 1, with higher values signifying superior expected quality. GMQE is coverage-dependent, meaning that a model covering only half of the target sequence is unlikely to achieve a score above 0.5. In contrast, QMEANDisCo evaluates the model irrespective of explicit coverage dependency. Figure 2a presents a graphical representation of the QMEANDisCo local quality estimate, offering insights into the model's local quality. Additionally, Figure 2b presents the Ramachandran Plot of the modelled structure, providing a visualization of energetically favoured regions for backbone dihedral angles of amino acid residues in the protein structure. Figure 2b delineates the contours of the favoured regions within the modelled structure.

Cavity detection on CDK4

The principal findings pertaining to the cavity detection of CDK4 are elucidated in Table 1, which encapsulates the number and characteristics of cavities identified in the target protein through CB-Dock2. Five prominent cavities (C1-C5) were successfully detected, and Table 1 provides comprehensive details regarding their size, volume, and other pertinent characteristics. Cavity 1 (C1) specifically exhibited a maximum Cavity Size of X, Y, and Z (28, -29, -65) and a Cavity Volume of 622 Å3, rendering it a focal point for molecular docking investigations. The sequence

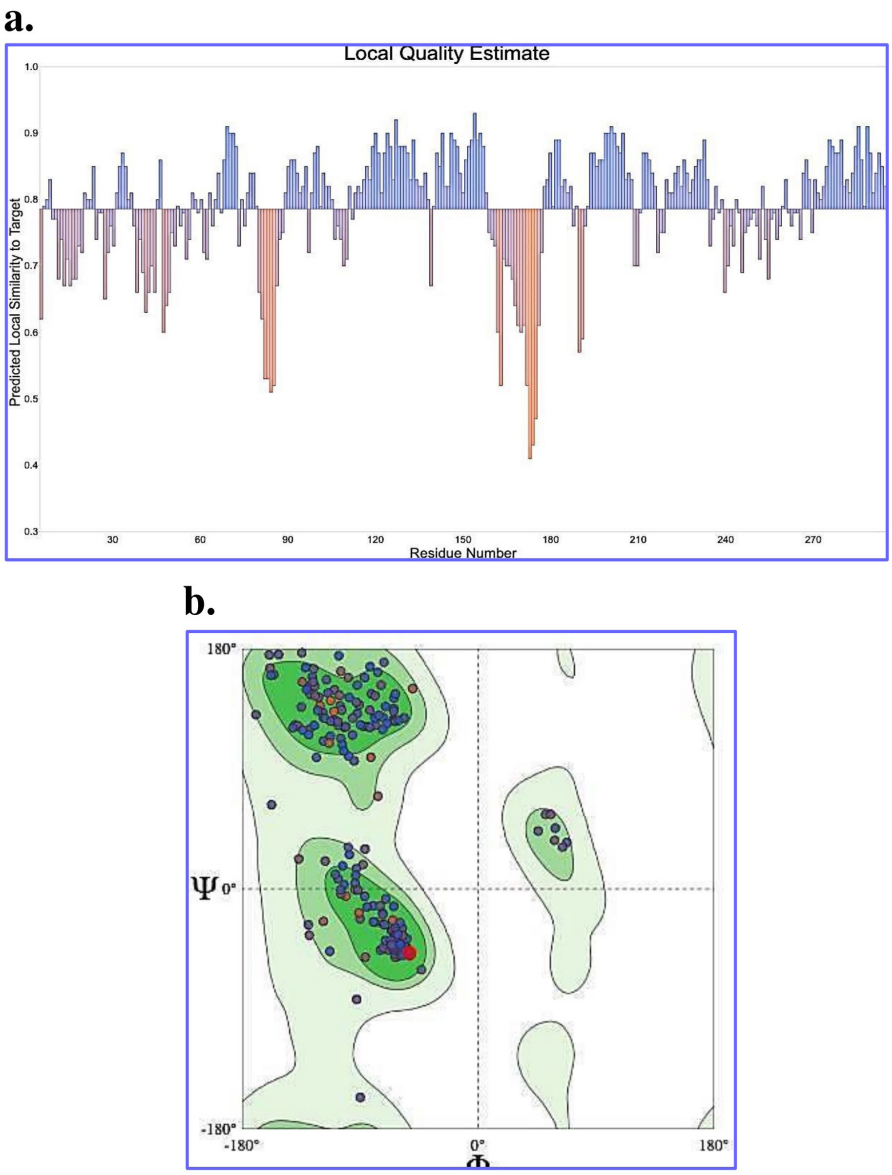


Figure 2: (a) QMEANDisCo local quality estimate graphically represents the model's local quality; (b) Ramachandran Plot visualizes energetically favored regions for backbone dihedral angles, with contours delineating these regions in the modelled structure.

representation of C1, C2, C3, C4, and C5 is depicted in Figure 3, while Figure 4 offers a structural visualization of all identified cavities (C1, C2, C3, C4, and C5) within CDK4.

Molecular docking revealed Betulinic Acid as potential inhibitor of CDK4

Upon screening a ligand library against CDK4, Betulinic Acid emerged as the most promising candidate, displaying a remarkable fitness of 98.97% and the highest predicted binding affinity. Subsequently, Betulinic Acid was selected as the top hit and subjected to molecular docking against the target protein CDK4. The visual representation of the molecular docking results for the CDK4:Betulinic Acid complex is depicted in Figure 5a-e. The docking analysis provided affinity scores and docked poses, revealing that Betulinic Acid exhibited a notable binding affinity score of -7.9 kcal/mol with CDK4. Figure 5a illustrates the cartoon presentation of the CDK4:Betulinic Acid complex, while Figure 5b presents the surface view of the same complex. Figure 5c depicts a 2D plot illustrating the amino acid residues of CDK4 engaged in various interactions with Betulinic Acid. The successful docking of Betulinic Acid into the deep binding cavity of CDK4 is evident from the docking pose, which formed several polar, hydrophobic, and other types of interactions with CDK4, as detailed in Table 2. The interaction of Betulinic Acid with the amino acid residues of CDK4 was further confirmed through HBPlot analysis, as shown in Figure 5d. Additionally, the binding affinity of the CDK4-Betulinic Acid complex was validated by redocking using SeamDock (https://bioserv.rpbs.univ-paris-diderot.fr/services/SeamDock/), with results presented in Figure 5e. Betulinic Acid demonstrated a binding affinity of

Figure 3: Sequence representation of cavities C1, C2, C3, C4, and C5.

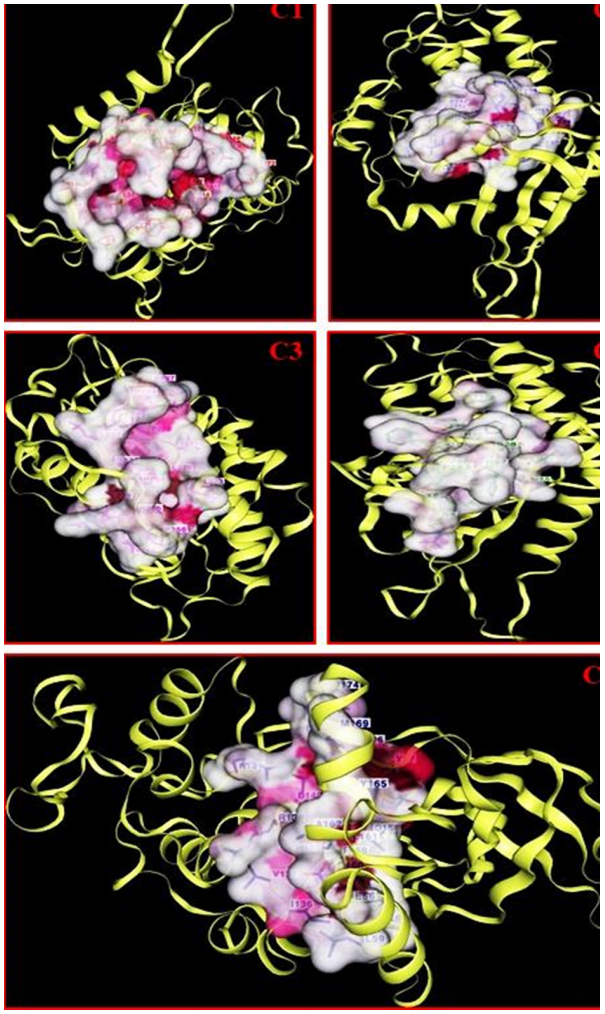


Figure 4: Structural visualization of all identified cavities (C1, C2, C3, C4, and C5) within CDK4.

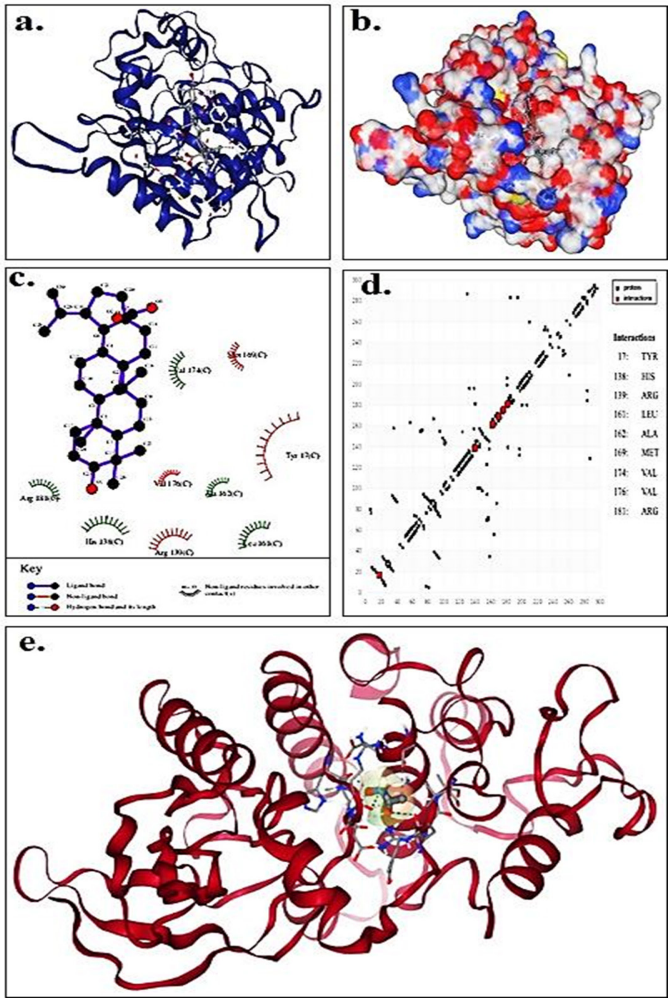


Figure 5: Visual representation of the CDK4: Betulinic Acid molecular docking complex. (a) cartoon presentation of the CDK4:Betulinic Acid complex; (b) Surface view of the complex; (c) 2D plot of the complex; (d) HBPlot analysis (e) Redocking validation of CDK4-Betulinic Acid binding affinity.

-6.5 kcal/mol with CDK4, further supporting its potential as a significant molecular candidate.

ADMET predictions, PASS analysis, and SwissTargetPrediction

ADMET predictions, PASS analysis, and SwissTargetPrediction collectively affirm the drug-like properties of Betulinic Acid. The summarized outcomes of ADMET predictions for Betulinic Acid are presented in Table 3, indicating that it complies with crucial pharmacokinetic criteria. This positions Betulinic Acid as a promising candidate for therapeutic development targeting CDK4 inhibition in cancer. PASS Analysis results, as detailed in Table 4, underscore favourable biological properties associated with Betulinic Acid, highlighting its relevance in various significant biological processes. Additionally, SwissTargetPrediction analysis not only confirms but also elaborates on the diverse biological properties linked to Betulinic Acid, as elucidated in Figure 6.

Concentration dependent cytotoxic effect of Betulinic Acid on MRC-9 normal lung cells

The results of concentration dependent cell toxicity assay are presented in Figure 7a. This assay showed no significant effect of Betulinic Acid on the viability of MRC-9 cells. Further, the IC_{50} value for Betulinic Acid was found to be 33.22 µM for A549 cancer cells and 41.12 µM for NCI-H460 cancer cells.

Time dependent cytotoxic effect of Betulinic Acid on MRC-9 normal lung cells

The outcomes of the time-dependent cell toxicity assay are delineated in Figure 7b. The findings indicate that the incremental exposure durations (24 hr, 48 hr, and 72 hr) of MRC-9 cells to the IC_{50} concentration of Betulinic Acid did not yield any discernible impact on their viability.

Expression of CDK4 in Betulinic Acid treated A549 and NCI-H460 lung cancer cells

The outcomes pertaining to the impact of the IC_{50} concentration of Betulinic Acid on the mRNA expression of CDK4 in A549 and NCI-H460 cancer cells are depicted in Figures 8a and 8b, respectively. The results manifest a noteworthy significant reduction in mRNA expression ($p<0.001$) of CDK4 in A549 and NCI-H460 cancer cells following treatment with the IC_{50} concentration of Betulinic Acid, as opposed to untreated cells.

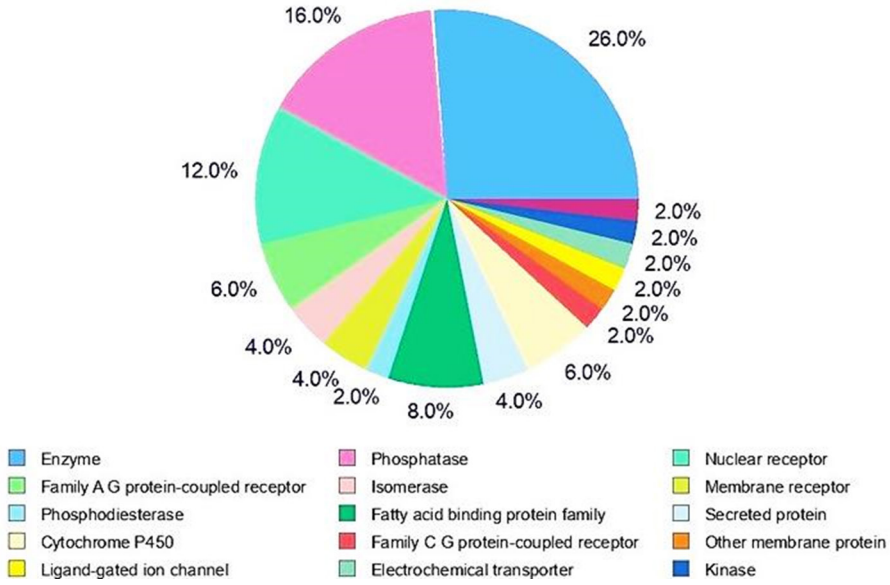


Figure 6: SwissTargetPrediction analysis showing diverse biological properties linked to Betulinic Acid.

Table 1: Detailed information on the size, volume, or other relevant characteristics of the detected cavities (C1-C5) on CDK4.

CurPocket ID	Cavity volume (Å^3)	Centre (x, y, z)	Cavity size (x, y, z)
C1	622	28, -29, -65	14, 14, 18
C2	463	34, -39, -64	14, 8, 12
C3	443	20, -67, -75	15, 9, 8
C4	395	37, -35, -53	11, 20, 10
C5	249	19, -42, -65	10, 8, 17

Table 2: Decomposed Interaction Energies in kcal/mol. Betulinic Acid formed several polar, hydrophobic and other types of the interactions with CDK4.

Polar	Hydrophobic	Other
ARG139 (0.9983)	LEU161 (-0.8301)	HIS138 (-1.1566)
	VAL174 (-0.7678)	ARG181 (-0.5611)
	ALA162 (0.2899)	
	VAL176 (0.0521)	
	MET169 (0.3225)	
	TYR17 (3.1937)	

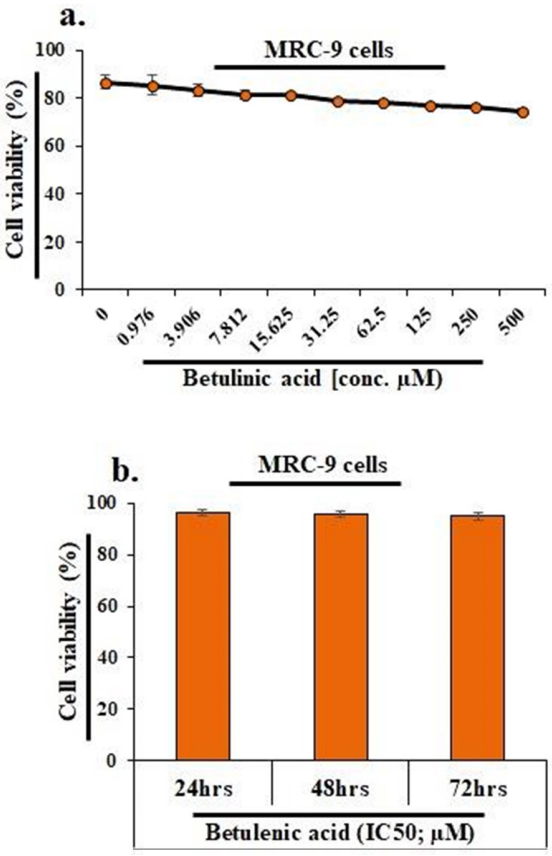


Figure 7: (a) Concentration dependent cell toxicity; (b) Time-dependent cell toxicity.

This finding underscores the inhibitory influence of Betulinic Acid on CDK4.

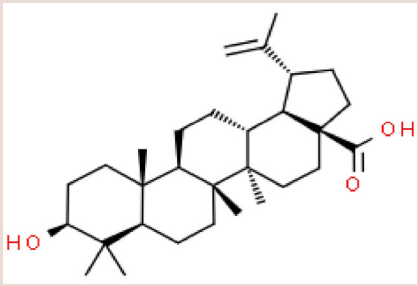
Enzyme inhibition assay showed inhibition of CDK4 by Betulinic Acid

The acquired kinase assay profile revealed a noteworthy significant reduction (p<0.001) in the kinase activity of CDK4, concomitant with an elevation in Betulinic Acid concentration (Figure 8c). This observation suggests the inhibitory impact of Betulinic Acid on the kinase activity of CDK4.

Table 3: In silico prediction of ADME and Toxicity properties for Betulinic Acid (BA).

	Properties																				
	Absorption	Distribution			Metabolism										Excretion		Toxicity				
Models	Intestinal absorption (human)	VDss (human)	BBB permeability	CNS permeability	CYP										Total clearance		AMES toxicity				
					Substrate		Inhibitor														
					2D6	3A4	1A2	2C19	2C9	2D6	3A4										
Unity	Numeric (% absorbed)	Numeric (log L/kg)	Numeric (Log BB)	Numeric (Log PS)	Categorical (yes/no)										Numeric (log ml/ min / kg)		Categorical (yes/no)				
Predicted values																					
BA	99.763	-1.18	-0.322	-1.343	NO	Yes	NO	NO	NO	NO	NO				0.116		NO				

Table 4: PASS (Prediction of Activity Spectra for Substances) analysis of Betulenic acid. Probability "to be active" was set at Pa>0,7.

Compound	Structure	Pa	Pi	Activity
Betulinic Acid		0.952	0.002	Hepatoprotectant
		0.923	0.003	Antiprotozoal (Leishmania)
		0.925	0.005	Antineoplastic
		0.891	0.008	Testosterone 17beta-dehydrogenase (NADP+) inhibitor
		0.881	0.007	Alkenylglycerophosphocholine hydrolase inhibitor
		0.848	0.008	Acylcarnitine hydrolase inhibitor
		0.835	0.003	Chemopreventive
		0.822	0.007	Apoptosis agonist
		0.815	0.004	Antineoplastic (lung cancer)
		0.814	0.004	Hepatic disorders treatment
		0.809	0.006	Oxidoreductase inhibitor
		0.804	0.003	Transcription factor stimulant
		0.804	0.003	Transcription factor NF kappa B stimulant
		0.805	0.004	Insulin promoter
		0.814	0.016	Antieczematic
		0.800	0.003	Antineoplastic (melanoma)
		0.794	0.005	Antineoplastic (colorectal cancer)
		0.798	0.010	Alkylacetylglycerophosphatase inhibitor
		0.789	0.005	Antineoplastic (colon cancer)
		0.768	0.002	Antinociceptive
		0.786	0.022	Mucomembranous protector
		0.762	0.005	Phosphatase inhibitor
		0.748	0.003	Beta glucuronidase inhibitor
		0.770	0.029	CYP2J substrate
		0.741	0.011	Antiinflammatory
		0.730	0.004	UGT1A4 substrate
		0.724	0.005	Antineoplastic (breast cancer)
		0.731	0.012	Membrane integrity antagonist
		0.724	0.014	Immunosuppressant
		0.709	0.004	Erythropoiesis stimulant
		0.708	0.004	Antineoplastic (ovarian cancer)
		0.704	0.003	Nitric oxide antagonist
		0.702	0.005	Cytoprotectant

Pa - Probability "to be active"; Pi - Probability "to be inactive".

DISCUSSION

This study investigates into the molecular understanding and potential therapeutic implications of Betulinic Acid as a CDK4 inhibitor. CDK4 plays a crucial role in cell cycle progression and is an attractive target for cancer therapy.[42,19] This study employs a comprehensive methodology to elucidate the inhibitory potential of Betulinic Acid against CDK4, aiming to bridge the gap in current knowledge and provide a foundation for future drug development in cancer. Betulinic acid is a pentacyclic triterpenoid found in the bark and other parts of several species of plants.[43] It exhibits anti-HIV, antimalarial, antineoplastic and anti-inflammatory properties.[44-46]

The high-resolution structure of CDK4 was modeled using Swiss-MODEL, showcasing a model with 100% sequence identity

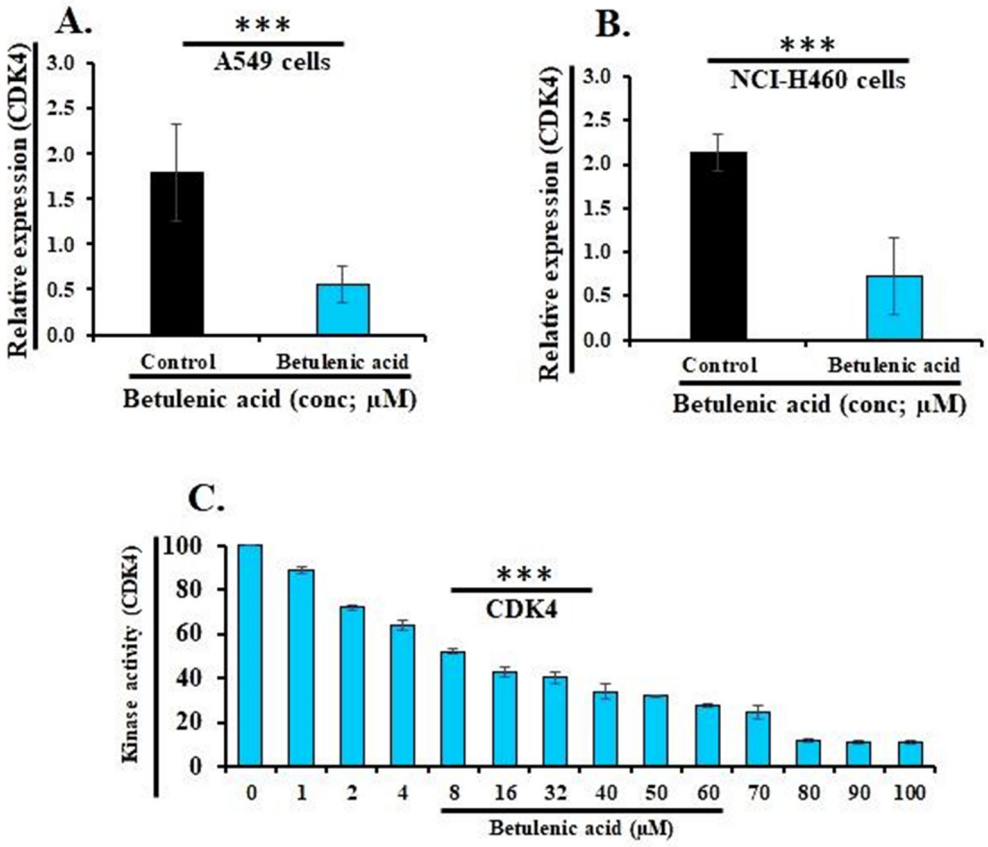


Figure 8: mRNA expression of CDK4 in (A) A549 cancer cells; (B) NCI-H460 cancer cells; (C) kinase activity of CDK4 with respect to increasing concentration of Betulinic Acid.

and coverage. The model's quality assessment revealed a GMQE score of 0.83 and a QMEANDisCo Global score of 0.79 ± 0.05, indicative of a robust and reliable structure. The Ramachandran plot further affirmed the model's structural integrity, aligning with energetically favored regions.

Cavity detection in molecular docking is a fundamental step that guides the identification of potential binding sites, facilitates accurate ligand binding predictions, and aids in the rational design of therapeutic molecules.[47,48] Cavity detection using CB-Dock2 unveiled five major cavities (C1-C5) on CDK4. Cavity 1 (C1) with a substantial size and volume became the focal point for molecular docking experiments. The detailed characterization of these cavities provides valuable insights into potential binding sites for small molecules like Betulinic Acid.[49]

Molecular docking is a powerful computational tool that accelerates the identification and development of therapeutic molecules by guiding the selection of lead compounds, optimizing their structures, and providing valuable insights into ligand-protein interactions.[50-52] Molecular docking studies identified Betulinic Acid as a top hit, displaying an impressive fitness of 98.97% and the highest predicted binding affinity. The docking poses illustrated successful interactions within the deep binding cavity of CDK4. Notably, Betulinic Acid formed diverse interactions, including polar and hydrophobic contacts, as corroborated by a 2D plot and HBPlot analysis. Redocking using SeamDock further validated the binding affinity, solidifying Betulinic Acid as a potent CDK4 inhibitor.

ADMET predictions provide valuable insights into the pharmacokinetic and safety profiles of therapeutic molecules.[53] By integrating these predictions into the drug discovery and development process, researchers can make informed decisions, optimize lead compounds, and increase the likelihood of success in bringing new drugs to market.[54,55] The drug-likeness properties of Betulinic Acid were confirmed through ADMET predictions, emphasizing its pharmacokinetic viability.

Prediction of Activity Spectra for Substances contributes to the identification and development of therapeutic molecules by predicting their biological activities, guiding lead compound selection, providing insights into mechanism of action, and supporting the exploration of multi-target drug design.[56] It serves as a valuable tool in the early stages of drug discovery, helping researchers make informed decisions and prioritize compounds with the greatest potential for therapeutic success. PASS analysis underscored favorable biological properties associated with

Betulinic Acid. SwissTargetPrediction is a valuable tool in drug discovery, aiding in target identification, mode of action exploration, lead optimization, and the assessment of safety and polypharmacological effects.[57] Its predictive capabilities contribute to rational drug design and facilitate the development of more effective and selective therapeutic molecules.[57,58,59] SwissTargetPrediction expanded on its diverse biological targets, strengthening its potential therapeutic applications.

Cytotoxicity assays are integral to the drug development process, providing valuable information on the safety and potential toxic effects of compounds.[60] They guide lead compound selection, contribute to mechanistic understanding, and play a key role in ensuring the overall safety of therapeutic molecules against specific cell lines.[61-64] Betulinic Acid exhibited concentration-dependent cytotoxic effects on A549 and NCI-H460 cancer cells, with an IC_{50} value of 33.22 µM and 41.12 µM, respectively. Importantly, no significant cytotoxicity was observed on MRC-9 normal lung cells. Time-dependent assays further emphasized the selectivity of Betulinic Acid towards cancer cells. The inhibitory impact of Betulinic Acid on CDK4 was validated at the molecular level, demonstrating a significant decrease in CDK4 mRNA expression in A549 and NCI-H460 cells. The enzyme inhibition assay corroborated these findings, revealing a concentration-dependent reduction in CDK4 kinase activity.

CONCLUSION

In conclusion, this comprehensive study provides a holistic understanding of CDK4 inhibition by Betulinic Acid, integrating structural insights with experimental validations. The identified interactions and inhibitory effects at the cellular and molecular levels highlight the potential of betulinic acid as a therapeutic drug with promise for targeting CDK4 in the treatment of cancer, especially lung cancer. The results reported here open up new avenues for preclinical and clinical research and add significant knowledge to the field of medication discovery.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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ABBREVIATIONS

CDKs: Cyclin-dependent kinases; **Rb:** Retinoblastoma protein; **LGA:** Lamarckian genetic algorithm; **DMEM:** Dulbecco's Modified Eagle Medium; **FBS:** Fetal bovine serum; **PBS:** Phosphate-buffered saline; **SD:** Standard deviation; **SEM:** Standard Error of the Mean; **IC_{50}:** Half-maximal inhibitory concentration.

SUMMARY

This study explores Betulinic Acid's potential as a therapeutic agent for targeting CDK4 in cancer treatment, including lung cancer. It provides structural insights and experimental validations, paving the way for further research.

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