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Small molecule 6"-O-Malonylglycitin inhibits ERK2 activity and promotes cytotoxicity in colon adenocarcinoma cells paving for the colon cancer treatment

Amany I. Alqosaibi[1,2]

Abstract

Background: Extracellular signal-regulated kinase 2 (ERK2), an important component of the MAPK signalling pathway, plays an critical role in the proliferation & survival of colon adenocarcinoma cells. Inhibition of ERK2 can interrupt these oncogenic pathways, offering a capable therapeutic strategy. Natural product-derived small molecules offer substantial potential in this context due to their favourable bioavailability & typically low toxicity.

Methods: This study examines the inhibitory effects of 6''-O-Malonylglycitin on ERK2 kinase activity & its cytotoxic effects on HCT-15 colon adenocarcinoma cells. The crystal structure of ERK2 (PDB ID: 2OJJ) was prepared for molecular docking studies, and 188 isoflavone natural compounds were screened using AutoDock Vina. Among these, 6''-O-Malonylglycitin exhibited the highest binding affinity (-9.3 kcal/mol) with ERK2, forming multiple hydrogen bonds & van der Waals interactions.

Results: The compound was evaluated for cytotoxicity by using an MTT assay, showing a dose-dependent reduction in cell viability with an IC50 of 15.33 µM. Time-dependent studies presented demonstrated significant decreases in cell viability up to 48 hours. Quantitative PCR indicated a significant reduction in ERK2 mRNA expression following treatment with 6''-O-Malonylglycitin. Kinase inhibition assay confirmed a concentration-dependent decrease in ERK2 activity.

Conclusion: The findings indicate that 6''-O-malonylglycitin effectively inhibits ERK2 kinase activity and exhibits potent cytotoxic effects against HCT-15 colon adenocarcinoma cells, suggesting its potential as a therapeutic agent for colon cancer.

Introduction

Mitogen-Activated Protein Kinases (MAPKs) are critical components of cellular signaling pathways that regulate numerous cellular processes, such as proliferation, differentiation, and apoptosis [1,2]. Among the MAPKs, four primary pathways-p38 MAPK, extracellular-signal-regulated kinase 1/2 (ERK1/2), Big MAP kinase 1 (ERK5), & c-Jun N-terminal kinase (JNK)-are particularly responsive to stress signals [3,4]. These pathways play important roles in cancer progression, including colon carcinogenesis [5, 6]. Understanding the distinct and overlapping activities of these MAPKs is critical for developing tailored cancer treatments. The p38 MAPK pathway plays a significant role in inflammation-associated colon carcinogenesis [7, 8]. It stimulates the production of inflammatory cytokines and chemokines, which influence the innate immune system and contribute to cancer initiation [9]. Inhibition of p38 MAPK can lead to the activation of the epidermal growth factor receptor (ErbB3) and subsequent activation of the MEK1/2-ERK1/2 pathway, regardless of the RAS or RAF proteins [10, 11, 12]. This emphasises the complicated interplay of many MAPK pathways and their implications in cancer progression.

Extracellular signal-regulated kinase (ERK1/2) activation is regulated by a complex network of tyrosine kinase receptors, cellular kinases, small GTPases, protein scaffolds, phosphatases, oncogenic mutations, and reactive oxygen species (ROS) [1,13,14,15]. Activation starts with cell membrane receptors that recruit adaptor molecules and tiny GTPase regulatory proteins, which subsequently stimulate GTP transfer to small GTPases like RAS [1,16]. Activated RAS triggers downstream kinases, including RAF, which additional transmits the signal to MEK1/2 and ERK1/2 [16,17]. Phosphorylation of ERK1/2 by MEK1/2 at specific residues (Thr202 and Tyr204) results in a conformational change that enables ERK1/2 to interact with downstream targets like ETS Like-1 protein (Elk-1) [6, 18, 19]. Phosphorylated ERK1/2 kinases promote various cellular processes, including growth, migration, survival, differentiation, & in some cases, cell cycle arrest, depending on their cellular localization and the downstream molecules they activate [20-21].

ERK1/2 are key intermediaries in signal transduction contributively to oncogene-induced senescence (OIS), transformation of primary cells, and regulation of ROS levels through modulation of superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) [1]. ERK1/2 signalling increases the expression of extracellular superoxide dismutase (SOD_3), which regulates O_2- levels at the cell membrane and controls its own expression [4]. This feedback loop plays a critical role in maintaining redox balance within the cell. In metastatic colon cancer, deregulation of the RAS-MEK1/2-ERK1/2 pathway due to RAS-associated mutations provides tumour cells with a selective growth & survival benefit, leading to acquired resistance to anti-EGFR therapy [22, 23, 24].

ERK2, a key component of the MAPK pathway, transduces extracellular signals to the nucleus, influencing gene expression and cellular responses [25, 6]. In normal cells, ERK2 activation is tightly regulated; however, in cancer cells, mutations and dysregulations in upstream components such as receptor tyrosine kinases, RAS, and RAF lead to constitutive ERK2 activation [6,1]. This persistent signalling promotes oncogenic processes, including proliferation, angiogenesis, metastasis, and resistance to apoptosis. The rationale for ERK2 inhibition is supported by the prevalence of ERK pathway dysregulation in cancers, preclinical efficacy, and potential for overcoming resistance [26,27]. Targeting ERK2 represents a promising strategy in cancer therapy, leveraging its pivotal role in oncogenic signalling.

Methods

Retrieval & preparation of ERK2

The crystal structure of ERK2 was retrieved from the RCSB PDB database, specifically under the PDB ID 2OJJ [28]. This initial structure was prepared following a conventional receptor procedure, as described [29]. The protein molecule's correctness and integrity were ensured using Swiss-PDB Viewer tools. These techniques enabled the detection and rectification of any structural defects in the downloaded crystal structure, such as missing atoms, improper bond ordering, or unresolved residues. The deformed geometries were then corrected using energy minimisation. This method entails altering atom locations and removing internal limitations from the protein structure, resulting in a more stable and realistic conformation. To finish the preparation, hydrogen atoms were added to the protein structure, along with Kollman United Atom Charges, which are required for precise molecular simulations. The fully produced and optimised structure was then saved in .pdbqt format using MGLTools, allowing for future computer analysis and molecular docking-based studies.

Retrieval and Preparation of the ligand molecules

In total, 188 isoflavone natural compounds were docked against ERK2. The structures of these ligand molecules were initially obtained in mol format from PubChem, a popular chemical database. To ensure compatibility with the docking program, these structures were translated to the PDB format using Open Babel, an open-source chemical toolkit that allows for the conversion of chemical file types. The

hydrogen atoms were then added to the ligand molecules using PyMOL, a molecular visualisation system for building and studying molecular structures. This phase is critical for correctly mimicking the physical and chemical properties of the ligands during the docking process. Swiss-PDB Viewer was used to save energy and produce the most stable configuration of the ligand molecules. This software facilitates the relaxation of ligand complexes to their lowest energy conformation, ensuring that the molecules are in a condition that closely mimics their natural form. Following energy minimisation, the structure of each ligand molecule was checked for any anomalies, such as missing atoms, improper bond ordering, or other structural flaws. This was accomplished using molecular modelling software, which includes capabilities for detecting and rectifying potential inaccuracies. Following rectification, the ligand molecules were allocated the appropriate atomic charges. The correct simulation of molecular interactions during docking requires accurate charge assignment. Finally, the optimised and rectified ligand structures were saved as PDBQT files using MGLTools. The PDBQT format is required for docking simulations in AutoDock Vina, a prominent molecular docking software. This format includes all of the necessary structural information, as well as atomic charges and rotatable bonds, making it appropriate for docking.

In Silico Docking Studies

We used AutoDock Vina for molecular docking simulations, focussing on blind docking of ligand molecules with the ERK2 protein, as reported in [32]. The docking method entailed setting the grid box dimensions for the X, Y, and Z coordinates to 56 Å, 79 Å, and 82 Å, respectively. The grid box was cantered at coordinates -45.22, 19.31, and 74.21. To achieve precise docking calculations, we kept the grid spacing at 1.00 Å and employed an exhaustiveness value of 8 to improve search thoroughness and accuracy. Following the docking simulations, we examined the ligand molecules' binding affinity to ERK2. The docked complexes were then visualised using PyMOL and the Discovery Studio Visualiser. These methods allowed us to examine the interaction patterns between ligand molecules and ERK2, revealing probable binding locations and the nature of the interactions, such as hydrogen bonds, hydrophobic interactions, and van der Waals forces. This comprehensive analysis was important for understanding the molecular basis of ligand-ERK2.

Chemicals and Cell Lines

6''-O-Malonylglycitin was purchased from MedChemExpress, Inc. and diluted in DMSO (dimethyl sulfoxide) from Sigma, USA, to prepare a stock solution at a concentration of 1 mg/mL. This preparation ensures that the compound is in the appropriate form for future studies. The HCT-15 colon cancer cell line was obtained from the National Centre for Cell Science (NCCS) in Pune, India. These cells were grown in RPMI 1640 media supplemented with 10% FBS (Foetal bovine serum) to provide growth factors and 1% antibiotics to prevent bacterial infection. OriGene Technologies, Inc., USA supplied the ERK2 protein (CAT#: AR09328PU-N), which was tagged with a His-tag for ease of purification and detection and has a purity of >95% by SDS-PAGE. This high-purity protein is required for precise and consistent experimental results.

Cell Culture & Cytotoxicity Assay

HCT-15 colon adenocarcinoma cells were grown in RPMI-1640 media supplemented with 10% FBS and 1% penicillin-streptomycin. The cells were incubated at 37°C in a humidified environment with 5% CO_2 until they reached confluence. This procedure maintains optimal growth conditions and prevents contamination. Once confluent, the cells were trypsinized, counted, and seeded into 96-well plates at a density of 5,000 to 7,000 cells per well. The cells were then allowed to adhere and grow for 48 hours. Following this incubation period, the cells were treated with various concentrations (0–1000 µM) of 6''-O-Malonylglycitin for 72 hours. Control wells were left untreated to serve as a baseline for comparison. After treatment, cell viability was assessed using the MTT assay. A 100 µl aliquot of MTT solution (5 mg/ml in phosphate-buffered saline) was added to each well. The plates were incubated at 37 °C for 3–4 hours, allowing the MTT to be metabolized by viable cells into purple formazan crystals. After incubation, the MTT solution was carefully removed, and 100 µl of DMSO was added to each well to dissolve the formazan crystals. The absorbance of the resulting solution was measured at 570 nm using a microplate reader, with a reference wavelength of 630 nm to correct for background absorbance. Cell viability was calculated as a percentage of the control wells using the formula: (Absorbance of treated cells / Absorbance of control cells) × 100.

To further assess the time-dependent effect of 6''-O-Malonylglycitin, a viability assay was performed in which HCT-15 cells were treated with the IC_{50} concentration of 6''-O-Malonylglycitin, defined as the concentration required to inhibit cell growth by 50%. Cell viability was measured at 24, 48, and 72 hours using the MTT assay, as described above.

mRNA expression in treated HCT-15

HCT-15 colon adenocarcinoma cells were treated through the IC_{50} concentration of 6''-O-Malonylglycitin

for a duration of 24 hours to investigate its effects on gene expression. Following the treatment, RNA was extracted from the cells via the Reagent (Trizol) provided by Thermo. The isolated RNA was subsequently reverse transcribed using Almanac's cDNA Synthesis Kit. The quantitative measurement of gene expression was performed using qRT-PCR (quantitative reverse transcription-polymerase chain reaction). This was accomplished utilising SYBR Green dye from G-Biosciences as a fluorescent reporter on an Applied Biosystems real-time PCR machine. The 2−ΔΔCT technique was used to measure gene expression levels in comparison to a control group. The primers used to amplify target genes were as follows: For ERK2, the forward primer sequence was 5′-GTGACCTCAAGCCTTCCAAC-3′, and the reverse primer sequence was 5′-AGAATGCAGCCTACAGACCA-3′ [1]. The forward primer sequence for GAPDH, which served as an internal control, was 5′-GAAGGTGAAGGTCGGAGTC-3′, while the reverse primer sequence was 5′-GAAGATGGTGATGGGATTTC-3′ [32].

Enzymatic assay

Next, we looked at how 6''-O-Malonylglycitin affected ERK2 kinase activity. ERK2 at a concentration of 2 µM was exposed to a range of 6''-O-Malonylglycitin concentrations, specifically 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, and 70µM. This setup was carried out on a 96-well microplate to guarantee high throughput and reproducibility. Each well contained ERK2 and a specific concentration of 6''-O-Malonylglycitin, and the plate was incubated for 1 hour at a regulated temperature of 25°C. Following the initial incubation period, each well received a reaction mixture containing 100 µM ATP and 10 mM MgCl2. The presence of ATP and MgCl2 is required for ERK2's kinase activity, which allows for phosphorylation. This mixture was incubated for a further 30 minutes to allow adequate time for the enzymatic reaction to proceed. To stop the processes, we used BIOMOL® reagent, which inhibits kinase activity. The resultant complex, which appears green due to the presence of malachite green reagent, was generated as a result of the release of inorganic phosphate during ATP hydrolysis. This green-colored complex was measured at 620nm with a plate reader to provide a quantitative estimate of kinase activity. The malachite green reagent is utilised specifically in this experiment to measure the inorganic phosphate produced, which acts as an indication of ERK2 activation. The absorbance measurements were compared to the baseline activity of native ERK2, which was not treated with 6''-O-Malonylglycitin and was recorded as 100% activity. This baseline served as a reference point for comparing the effects of different doses of 6''-O-Malonylglycitin on ERK2 kinase activity.

Statistical analyses

Data are presented as Mean ± SE. Data was analysed using SPSS. The t-test was employed to compare the mean values of control and treated group with respect to assess the Effect of 6''-O-Malonylglycitin on HCT-15 colon adenocarcinoma cells in a time dependent manner, effect on mRNA expression, and kinase activity. $p<0.05$ was considered as statistically significant difference.

Results

Molecular docking examination

The binding affinities of the top five hits are shown in Table 1. The maximum binding affinity was found between 6''-O-Malonylglycitin and ERK2, at -9.3 kcal/mol. Figure 1A represents the Chemical structure of Ligand molecule "Malonylglycitin" and Figure 1B-D depicts the interactions between 6''-O-Malonylglycitin and ERK2. Specifically, 6''-O-Malonylglycitin established many typical hydrogen bonds with the ERK2 amino acid residues PHE352, ALA90, and ASP98. In addition to these hydrogen bonds, 6''-O-Malonylglycitin engaged in various van der Waals and other interactions with several amino acid residues, including ARG22, ASP86, GLY20, ILE88, PRO354, GLN353, PRO91, ARG89, TYR100, VAL99, MET96, LYS97, VAL16, GLN95, ASP18, PRO21, and TYR23.

Name of the ligand	Target protein	Binding Energy (kcal/mol)	pKi
6''-O-Malonylglycitin	ERK2	-9.3	4.47
Genistein	ERK2	-7.5	3.53
Prunetin	ERK2	-7.1	3.21
Calycosin	ERK2	-6.0	3.1
Ononin	ERK2	-5.6	3.0

Table 1: Binding affinities of top five ligand molecules with the target protein, ERK2. Results indicated that 6''-O-Malonylglycitin has the highest binding affinity with the target protein.

Cytotoxic effect of 6''-O-Malonylglycitin on HCT-15 colon adenocarcinoma cells

Figure 3 A shows the findings of the concentration-dependent cell toxicity assay, which shows how different doses of 6''-O-Malonylglycitin affect the viability of HCT-15 colon cancer cells. The assay revealed a clear dose-dependent reduction in cell viability, indicating that larger concentrations of 6''-O-Malonylglycitin are associated with greater cytotoxicity. This result suggests that 6''-O-Malonylglycitin has a strong inhibitory effect on the growth and survival of these cancer cells. Furthermore, the half-maximal inhibitory concentration (IC_{50}) of 6''-O-Malonylglycitin in HCT-15 colon cancer cells was calculated as 15.33µM. The comparatively low IC_{50} value demonstrates 6''-O-Malonylglycitin's ability to produce cytotoxic effects on these cancer cells,

implying that it has potential as a therapeutic agent in the treatment of colon adenocarcinoma.

Figure 3B shows the findings of the time-dependent cell toxicity assay. The assay demonstrates that continuous exposure of HCT-15 colon adenocarcinoma cells to the IC_{50} concentration of 6''-O-Malonylglycitin dramatically reduces cell viability over time. The study found a significant reduction in cell viability up to 48 hours of exposure ($p<0.001$). However, there was no significant difference in cell viability between the 48- and 72-hour exposure periods. This implies that the cytotoxic effects of 6''-O-Malonylglycitin peak after 48 hours, with no additional rise in cell death after that point.

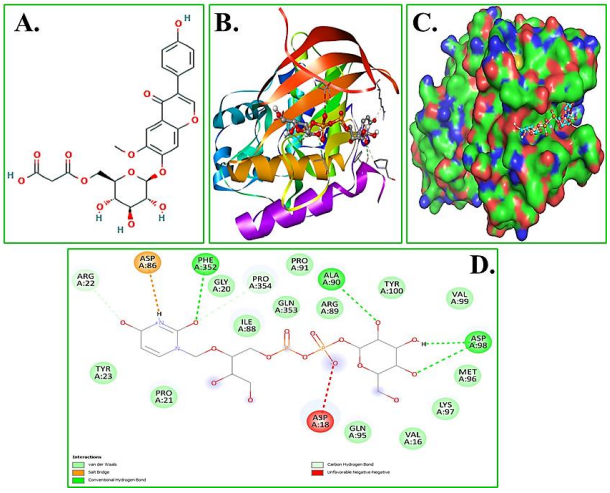

Figure 1: Molecular docking of 6''-O-Malonylglycitin with ERk2. (A) Chemical structure of Ligand molecule "Malonylglycitin" (B) Cartoon representation of Malonylglycitin-ERK2 complex, (C) Surface view representing binding of 6''-O-Malonylglycitin within deep groove of ERK2, and (D) 2D docked complex showing interaction of 6''-O-Malonylglycitin with various amino acid residues of ERK2.

Swiss Target Prediction

The outcomes of the SwissTargetPrediction examination are presented in Figure 2. The study revealed that 6''-O-Malonylglycitin is associated with a variety of biological properties. Specifically, the data indicate that this compound interacts with multiple biological targets, suggesting its potential involvement in several biochemical pathways. These interactions underscore the compound's diverse biological activities, which may include anti-inflammatory, antioxidant, and anticancer effects. The detailed results, as illustrated in Figure 2, highlight the broad spectrum of potential therapeutic applications of 6''-O-Malonylglycitin.

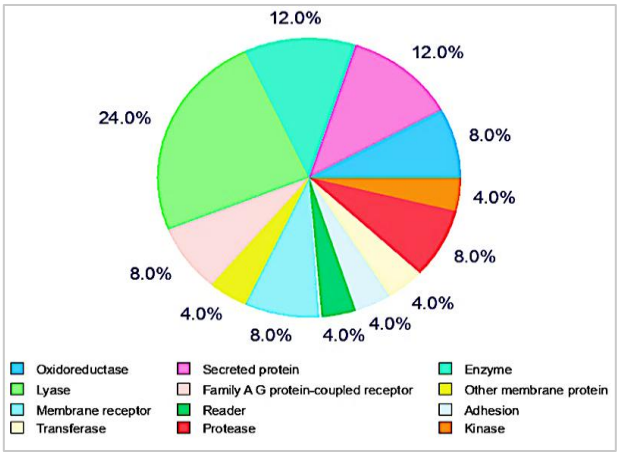

Figure 2: Swiss Target Prediction analysis. 6''-O-Malonylglycitin are linked to various biological molecules associated with diverse biological processes.

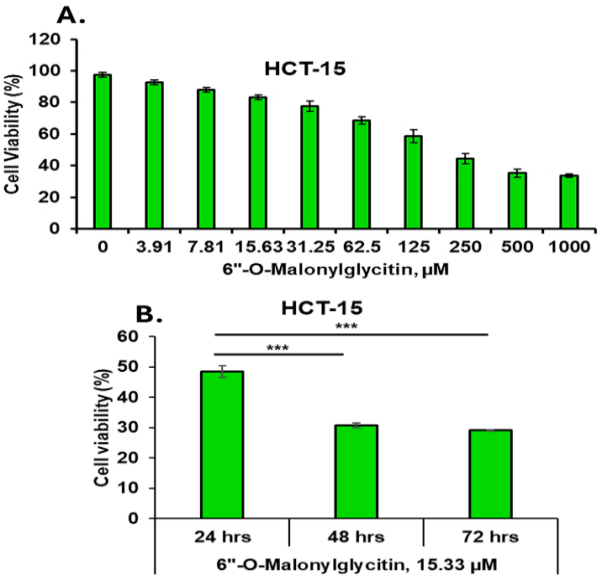

Figure 3 (A): Effect of 6''-O-Malonylglycitin on HCT-15 colon adenocarcinoma cells in a concentration dependent manner. (B) Effect of 6''-O-Malonylglycitin on HCT-15 colon adenocarcinoma cells in a time dependent manner.

Expression of ERK2 in 6''-O-Malonylglycitin treated HCT-15 colon adenocarcinoma cells

The findings provided in Figure 4A show that the IC_{50} concentration of 6''-O-Malonylglycitin has a highly significant impact on the mRNA expression levels of ERK2 in HCT-15 colon cancer cells, with a p-value less than 0.001. This result shows that treatment with 6''-O-Malonylglycitin significantly reduces ERK2 mRNA expression in these cancer cells. The study found that HCT-15 colon adenocarcinoma cells treated with 6''-O-Malonylglycitin had considerably reduced mRNA expression levels ($p<0.001$) compared to the untreated control group. This decrease in mRNA expression indicates that 6''-O-Malonylglycitin may effectively

downregulate ERK2, which could contribute to its potential therapeutic benefits on HCT-15 colon cancer cells.

Kinase inhibition assay showed inhibition of ERK2 by 6''-O-Malonylglycitin

The kinase assay results (Figure 4B) revealed a highly significant decrease in the activity of the kinase ERK2, with a p-value of less than 0.001, as the quantity of 6''-O-Malonylglycitin was raised. This substantial statistical significance emphasises the strength of the observed effect. The results clearly show that 6''-O-Malonylglycitin has a strong inhibitory effect on the kinase activity of ERK2. The decrease in kinase activity is consistent with increased levels of 6''-O-Malonylglycitin, implying a direct link in which 6''-O-Malonylglycitin functions as an ERK2 inhibitor. This observation is significant because it suggests that 6''-O-Malonylglycitin has the capacity to modify ERK2 activity, which could have important implications for understanding its involvement in cellular signalling pathways and potential therapeutic uses.

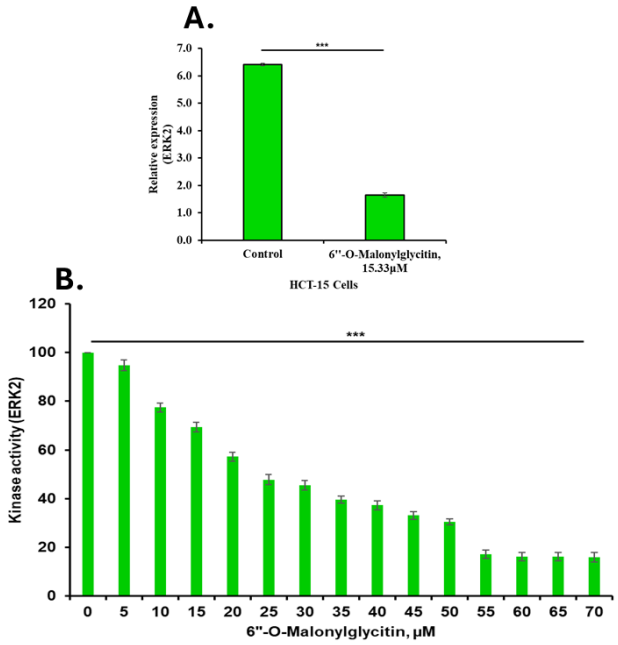

Figure 4 (A): Effect of the IC50 dose of 6''-O-Malonylglycitin on ERK2 mRNA expression. (B) Kinase activity of ERK2 with respect to increasing concentration of 6''-O-Malonylglycitin.

Discussion

In recent years, there has been a major increase in the study of natural chemical molecules for therapeutic interventions in various cancers [33–35]. This work investigates the inhibitory potential of the isoflavone compound 6''-O-Malonylglycitin against extracellular signal-regulated kinase 2 (ERK2) and its implications for the treatment of colon cancer. The study's findings emphasise the compound's strong binding affinity, significant influence on cell survival, and significant inhibition of ERK2 at both the mRNA and kinase activity levels.

Molecular docking of target proteins and ligands is important in drug development because it predicts a prospective medication's binding affinity and interactions with its target [36, 37]. It aids in the identification of lead compounds, optimisation of drug design, and prioritisation of candidates, hence expediting the creation of effective and specific therapies [38,39,40]. The molecular docking investigations demonstrated that 6''-O-Malonylglycitin had a strong affinity for ERK2, with a binding energy of -9.3 kcal/mol. The molecule forms multiple typical hydrogen bonds with important amino acid residues, including PHE352, ALA90, and ASP98. Furthermore, van der Waals and other molecular interactions with residues including ARG22, ASP86, GLY20, ILE88, PRO354, GLN353, PRO91, ARG89, TYR100, VAL99, MET96, LYS97, VAL16, GLN95, ASP18, PRO21, and TYR23 were observed. These interactions are essential for the stability and specificity of 6''-O-Malonylglycitin binding to ERK2, which increases its efficacy as an inhibitor. SwissTargetPrediction analysis is useful for drug discovery since it identifies potential biological targets of small molecules, which aids in the selection of compounds with therapeutic potential [41]. Predicting interactions with individual proteins improves drug design, optimises screening procedures, and speeds up the identification of promising therapeutic candidates [42]. In this work, SwissTargetPrediction analysis revealed a wide range of biological features associated with 6''-O-Malonylglycitin, indicating that it has promise for a variety of therapeutic uses.

Cell viability assays, such as the MTT assay, are important in drug discovery because they assess drugs' lethal effects on cultured cells [43, 44]. These tests quantify metabolic activity to evaluate chemical efficacy, dose-response relationships, and selectivity, providing critical data for evaluating potential therapeutic medicines in preclinical research [45,46]. In vitro experiments showed a considerable impact on the viability of HCT-15 colon cancer cells (IC_{50} = 15.33µM). Time-dependent cell toxicity experiments demonstrated a significant reduction in cell viability over 48 hours at the IC_{50} concentration, indicating that the chemical is effective in a persistent inhibitory role. However, there was no significant difference in cell viability between the 48- and 72-hour exposure periods. The observed plateau in cytotoxicity after 48 hours suggests that 6''-O-Malonylglycitin exerts its maximal effect within this timeframe. This may indicate saturation of its cytotoxic mechanism or

activation of cellular defence responses. Further studies are needed to explore whether resistance mechanisms or compound stability contribute to this time-dependent effect.

Assessing mRNA expression levels of target proteins is critical in drug development and cancer treatment because it gives information about gene regulation and possible therapeutic targets [47]. Understanding the impact of drugs on gene expression and tumour growth aids in the identification of critical molecular changes, the assessment of medication efficacy, and the optimisation of treatment regimens [48, 49]. The study found a statistically significant decrease in ERK2 mRNA expression in HCT-15 cells treated with 6''-O-Malonylglycitin, showing that the substance effectively downregulates ERK2 transcriptionally. This reduction is critical because ERK2 is a key player in the MAPK/ERK signalling pathway, which is frequently dysregulated in malignancies, including colon adenocarcinoma [4, 50]. The significant downregulation of ERK2 mRNA expression in HCT-15 cells upon treatment with 6''-O-Malonylglycitin, suggests a targeted effect on the ERK2 signalling pathway. However, further studies are warranted to determine whether this reduction is specific to ERK2 or extends to other components of the MAPK/ERK pathway or related signalling networks. Future studies should assess the expression of upstream kinases (e.g., MEK1/2) and downstream targets, as well as other MAPKs like ERK1, JNK, and p38, to clarify the specificity and broader implications of 6''-O-Malonylglycitin's molecular effects. Furthermore, increasing concentrations of 6''-O-Malonylglycitin resulted in a significant decrease in ERK2 kinase activity, indicating the compound's direct inhibitory effects on ERK2 enzymatic function.

Comparing these findings to earlier research on ERK2 inhibitors and their impact on colon cancer provides a more complete picture of 6''-O-Malonylglycitin's potential. For example, studies on synthetic ERK2 inhibitors like SCH772984 and BVD-523 have shown significant anti-proliferative properties in a variety of cancer cell lines, including colon cancer [51, 52]. These inhibitors typically have IC_{50} values in the low micromolar range, which is similar to the IC_{50} value of 6''-O-Malonylglycitin found in this study. However, unlike synthetic inhibitors, 6''-O-Malonylglycitin, as a natural molecule, may have a higher safety profile and lower toxicity, which is beneficial for therapeutic applications.

Furthermore, prior research on isoflavone compounds, such as genistein and daidzein, have demonstrated their ability to suppress cancer cell growth and modulate signalling pathways [53, 54, 55]. Genistein, for example, has been shown to limit the proliferation of several cancer cells, particularly colon cancer cells, by targeting multiple signalling pathways, including the MAPK/ERK pathway [56]. The efficacy of 6''-O-Malonylglycitin in lowering ERK2 activity and expression is consistent with previous findings, implying that this molecule could function as a powerful natural inhibitor with selective action on the MAPK/ERK pathway.

The strong inhibition of ERK2 by 6''-O-Malonylglycitin, as well as its effect on colon adenocarcinoma cell survival, suggest that it has therapeutic potential. The compound's capacity to downregulate ERK2 at the mRNA level while inhibiting its kinase activity suggests a dual mechanism of action, which is beneficial for therapeutic efficacy. Furthermore, the natural origin of 6''-O-Malonylglycitin may provide advantages in terms of less side effects and improved patient tolerance when compared to synthetic inhibitors.

With respect to clinical relevance of the findings, The shown inhibitory actions of 6''-O-Malonylglycitin on ERK2 indicate prospective clinical significance, particularly in malignancies characterised by abnormal MAPK/ERK signalling. This drug, which targets ERK2 at various levels—transcriptional regulation, kinase activity suppression, and persistent binding—may provide a complex treatment strategy. Its ability to diminish cancer cell viability adds to its promise as an anticancer drug. These data present a compelling case for doing preclinical investigations in animal models to evaluate pharmacokinetics, effectiveness, and toxicity. Ultimately, 6''-O-Malonylglycitin could be used as a lead chemical in the development of tailored treatments for ERK2-dependent cancers, meeting an unmet need in precision oncology.

While our in vitro data significantly support the therapeutic promise of 6''-O-Malonylglycitin as an anti-cancer drug that targets ERK2, the importance of in vivo validation cannot be emphasised. In vivo studies are required to validate the compound's pharmacokinetics, bioavailability, toxicity profile, and overall efficacy in a physiological setting. They would shed light on how 6''-O-Malonylglycitin interacts with the complex tumour microenvironment and whether the observed ERK2 inhibition results in meaningful therapeutic benefits in living beings. As a result, future research should incorporate animal models to evaluate these elements and support the compound's clinical relevance as a cancer therapy option.

In conclusion, the results of cell survival tests, mRNA expression analysis, kinase activity tests, and binding affinity investigations taken together clearly suggest that 6''-O-Malonylglycitin has therapeutic potential as an anti-cancer drug that targets ERK2. The compound's capacity to inhibit ERK2 kinase activity,

decrease cell viability, downregulate ERK2 mRNA expression, and establish persistent connections with ERK2 points to a complex mode of action that may be used in cancer treatment. Future research should concentrate on clarifying the molecular processes behind 6''-O-Malonylglycitin's ERK2 inhibitory actions. A more comprehensive understanding of the compound's therapeutic potential would be possible by looking into how it affects other elements of the MAPK/ERK circuit as well as how it behaves in in vivo models. Furthermore, investigating structural changes to 6''-O-Malonylglycitin to improve its potency and specificity may open the way for the development of more potent ERK2 inhibitors.

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Conflict of Interest

The authors declare that they have nothing to disclose.

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