JOURNAL OF COMPLEMENTARY MEDICINE RESEARCH

Journal of Complementary Medicine Research, ISSN: 2146-8397 Vol. 15, No. 1, 2024(pp. 71-80)

RESEARCH ARTICLE WWW.JOCMR.COM

Dihydrosinularin Induces Apoptosis, G2 Cell Cycle Arrest and DNA Damage in Human Non-Small Cell Lung Cancer Cells

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ABSTRACT

The objective of this study was to analyze the antiproliferative activity of Dihydrosinularin, a cembranolide isolated from the soft coral Sinularia flexibilis against cancer CL1-5 cells and to determine the mode of cancer cytotoxicity. An Thiazolyl Blue Tetrazolium Blue (MTT) assay was used to determine the cytotoxic effect of dihydrosinularin. Subsequently, apoptosis and cell cycle analyses were performed by using flow cytometry. Apoptosis, G2/M cell cycle control and DNA-damage response protein expression were assessed using Western blotting. Using the MTT method, we discovered that dihydrosinularin dramatically reduced growth of CL1-5 cells. By using a flow cytometry assay, it was demonstrated that dihydrosinularin caused G2/M cell cycle arrest and dependent apoptosis in CL1-5 cells. JC-1 fluorescence labeling was also used to show the reduction of mitochondrial membrane potential (MMP) in dihydrosinularin-treated cells. In addition, dihydrosinularin influenced the expression levels of apoptotic and G2/M cell cycle arrest-related proteins. Moreover, the expression of γ-H2AX considerably, showing that dihydrosinularin caused DNA double-strand breaks. The ATM/Chk2 DNA damagee response signaling pathway was then activated. This study showed that dihydrosinularin promoted apoptosis, G2/M arrest, and DNA damage pathways, which could be important for cytotoxicity in human non-small cell lung cancer cells.

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How to cite this article: Lin C, Chao Y, Chiang Y, Yang T, Chang Y, Yang T, Chang K, Lin S (2024), Dihydrosinularin induces apoptosis, G2 cell cycle arrest and DNA damage in human non-small cell lung cancer cells. Journal of Complementary Medicine Research, Vol. 15, No. 1, 2024 (pp. 71-80).

KEYWORDS:
Dihydrosinularin;
non-small
cell lung cancer;
apoptosis;
cell cycle;
DNA damage

ARTICLE HISTORY:
Received: Oct 20, 2023
Accepted: Nov 25, 2023
Published: Dec 22, 2023

DOI:
10.5455/jcmr.2024.15.01.12

INTRODUCTION

With 1.8 million deaths due to cancer-related causes worldwide in 2020, lung cancer continues to be a serious public health concern [Sung *et al.*, 2021]. Small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) make up the majority of lung cancer cases, accounting for 85% of all lung cancer cases [Bareschino *et al.*, 2011]. The 5-year overall survival after diagnosis is still quite low, especially in advanced patients, despite improvements in clinical diagnosis and treatment [Sung *et al.*, 2021; Herbst *et al.*, 2018]. This is due to the growing resistance of targeted therapies. Because of this, it is becoming more and more important to come up with new drugs and ways to treat NSCLC in order to make treatment more effective and improve patients' chances of survival.

Dysregulated apoptosis causes several disorders, including cancer. This biological process involves caspase-mediated programmed cell death and is characterized by chromatin condensation, nuclear fragmentation, and cell shrinkage [Reed, 2000]. Apoptosis may emerge via an extrinsic signaling pathway (death receptor pathway) initiated by transmembrane receptor-mediated contacts or an intrinsic signaling pathway (mitochondrial pathway) produced by non-receptor-mediated stimuli, such as radiation, toxins, hypoxia, or free radicals [Elmore, 2007]. Since cancer cells have messed up apoptosis, causing apoptosis is a good way to treat cancer [Pfeffer & Singh, 2008].

When DNA is broken, G2/M cell cycle arrest occurs, preventing cells from beginning mitosis and allowing for DNA repair in order to maintain genomic stability [Boohaker & Xu, 2014; Hsia *et al.,* 2016; Yan *et al.,* 2014]. The ATM gene encodes a serine/threonine protein kinase that is involved in DNA repair in a variety of cancers [Hsia *et al.,* 2016; Yan *et al.,* 2014]. ATM, as a product of the ATM gene, has the ability to autophosphorylate and is critical to the DNA damage checkpoint, the repair of damaged DNA, and apoptosis [Boohaker, Xu, 2014]. ATM, along with other proteins such as p53 and checkpoint kinase (CHK2), is involved in cell cycle arrest in response to DNA damage [Stark & Taylor, 2006.].

Soft corals contain numerous bioactive natural chemicals [Rodrigues *et al.,* 2019] with anticancer properties [Huang *et al.,* 2018; Lai, *et al.,* 2022]. Dihydrosinularin was discovered first in the soft coral S. flexibilis [Sanduja *et al.,* 1986]. (9E)-13-hydroxy-4,9,13,17-tetramethyl-5,15-dioxatricyclo [1 2.3.1.04,6] octadec-9-en-16-one is the IUPAC designation for dihydrosinularin [Kim *et al.,* 2019]. Dihydrosinularin is known to have antioxidant [Wang *et al.,* 2021] and anti-inflammatory [Su & Wen, 2011] biological activities. Recently, a few studies have pointed out that dihydrosinularin has anti-cancer potential. Wang et al. [Wang, *et al.,* 2021] discovered that dihydrosinularin is cytotoxic to breast MDA-MB-231 cells, lung H1299 cancer cells, liver HA22T/VGH cancer cells, and lymphocytic leukemia P-388 cells [Sanduja, et al., 1986]. In addition, Yang et al., study indicated that dihydrosinularin exhibits an antiproliferation effect via inducing G2/M cell cycle arrest and apoptosis via the ROS/DNA damage axis on oral cancer cells without cytotoxicity towards non-malignant oral cells [Yang, *et al.,* 2021].

However, although there are some studies pointing out that dihydrosinularin has a cytotoxic effect on cancer cells, there is only one study on oral cancer to explore its possible mechanism of action [Yang, *et al.,* 2021]. Therefore, exploring the efficacy and mechanism of dihydrosinularin on different cancer cells is still an important research task to confirm its anticancer potential. Thus, in this study, the anticancer effects of dihydrosinularin were evaluated on human NSCLC cell lines. The possible molecular mechanisms responsible for its anticancer activity were also investigated.

MATERIAL AND METHODS

Cell lines and cell culture

The human lung adenocarcinoma cell line, CL1-5, and human lung squamous carcinoma cell line were provided by Dr. Jeremy J W Chen (National Chung Hsing University, Taiwan). H226 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). HFL1 (BCRC Number: 60299) normal human lung fibroblast was purchased from the Food Industry Research and Development Institute (Hsinchu City, Taiwan). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F-12 Nutrient Mixture, which was supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, penicillin, and streptomycin. (FBS), L-glutamine (2 mM), and penicillin (100 U/ml)-streptomycin (100 mg/ml) were obtained from Gibco/Life (Carlsbad, CA, USA). The cultures were kept at 37°C in a humidified incubator containing 5% CO_2.

MTT assay

Cells were grown in 24-well (5×10^4/well) plates and treated for 24 hours with various doses of dihydrosinularin. Each well was then filled with 300 □L of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, St. Louis, MO, USA) solution (0.5 mg/mL final concentration). After 4 hours of incubation, the supernatant was aspirated and 600 µL of DMSO was added. The absorbance at 570 nm was measured using a microplate reader (TECAN, Durham, NC, USA). The absorbance of dihydrosinularin-treated cells relative to DMSO-treated cells is shown. GraphPad Prism software (Version 8.0, San Diego, CA, USA) was used to calculate the 50% inhibitory concentration (IC50).

Annexin V/PI Apoptosis Detection Assay

The method of cell death was determined using the Annexin V-FITC apoptosis detection kit (Biolegend, San Diego, CA, USA), and the analysis was carried out in accordance with the manufacturer's protocol. The cells were planted in a 6-well plate at a density of 2×10^5 cells/well and treated for 24 hours with dihydrosinularin. The cells were extracted and resuspended in 1× binding buffer after centrifugation (1000 rpm for 5 minutes). To stain the cells, 5 µL Annexin V-FITC (90 µg/mL) and 10 µL PI (0.5 µg/mL) were added to each solution and incubated at room temperature for 10 minutes in the dark. Apoptotic cells were detected using a BD Accuri C5 cytometer, and the data was analyzed with BD Accuri C6 Software 1.0.264.21.

Caspase-3, -8, and -9 activity assay by flow cytometry

CaspGLOW fluorescein active caspase staining kits (Biovision, Milpitas, CA, USA) were used to quantify human caspase-3, -8, and -9 activities according to the manufacturer's instructions. The cells were planted in a 6-well plate at a density of 2×10^5 cells/well and treated for 24 hours with dihydrosinularin. Flow cytometry on an Accuri C5 cytometer was used to assess the intensity of the fluorescence (BD Biosciences).

Western blot assay

The cells were planted at a density of 2×10^5 cells per well in a 6-well plate and treated with dihydrosinularin for indicated times. Following each treatment, cells were lysed in RIPA buffer, and protein preparations (15-40□□g) were separated by SDS-PAGE and transferred to Immobilon-P Transfer Membranes (Merck Millipore, Burlington, MA, USA). Incubation of membranes with particular primary antibodies included cleaved PARP (cell signaling), Bcl-2, Bax, phospho-Cdc25C (Ser-216), cdc25c, Cdc2 (Tyr-15), cdc2, cyclin B1, GAPDH, p-ATM (Ser1981), ATM, p-Chk2 (Ser19), CHK2, p-H2A.X, p-ATR (Ser428), ATR,□□p-Chk1 (Ser317), CHK1. Horseradish peroxidase conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) were used to detect the bands overnight at 4 °C, and the enhanced chemiluminescence detection kit reagent (Millipore, Burlington, MA, USA) was used to detect the bands, and the bands were imaged using a Hansor Luminescence Image System (Taichung, Taiwan). As a loading control, GAPDH was used. The intensity of the bands was measured using the NIH ImageJ 1.47 tool for Windows (Bethesda, MD, USA).

Evaluation of mitochondrial membrane potential

MMP alterations were assessed using the fluorescent probe JC-1. In brief, the cells were planted in a 6-well plate at a density of 2×10^5 cells/well and treated for 24 hours with dihydrosinularin. Cells were treated in the dark for 30 minutes with JC-1 dye (Invitrogen Life Technologies, Carlsbad, CA, USA) and then were resuspended in 500 ¤L PBS after being washed twice with PBS and examined using flow cytometry on an Accuri C5 cytometer (BD Biosciences).

Flow Cytometric Analysis of Cell Cycle Distribution

The cells were planted in a 6-well plate at a density of 2×10^5 cells/well and treated for 24 hours with dihydrosinularin. After that, the cells were collected and washed twice with PBS. The resuspended cells were then fixed overnight in 70% ethanol at 20 °C. Following that, the cells were stained in PI solution containing 1 mL of PBS, 50 ¤g/mL PI (Sigma-Aldrich, St. Louis, MO, USA), and 100 ¤g/mL /mL RNase A (Sigma-Aldrich, St. Louis, MO, USA) for 30 minutes in the dark at room temperature. An Accuri C5 cytometer was used to identify and analyze cell cycle distribution (BD Biosciences).

Statistical Analysis

The data are presented in triplicate as means standard deviation (SD). Multiple experimental groups were compared using one-way ANOVA (GraphPad Prism Version 5.0). (San Diego, CA, USA). A probability (p) value of 0.05 was regarded as the level of statistical significance.

RESULT

Dihydrosinularin inhibited the viability of CL-15 and H226 Cells

The MTT assay was used to determine the effects of dihydrosinularin (Fig. 1A) on cell viability in CL1-5, H226 human NSCLC cancer cell lines and normal human lung fibroblasts (HFL-1). The findings indicated that the chemical could reduce cell viability in a dose-dependent way and was significantly more toxic to CL1-5 and H226 cancer cells than to normal fibroblasts (Fig 1B). The IC50 values of andrographolide for CL1-5 cells, H226 and HLF-1 at 24 h, were 10.7± 5.2 μM, 15.3 ± 4.5 μM and 39.3 ± 7.5 μM respectively.

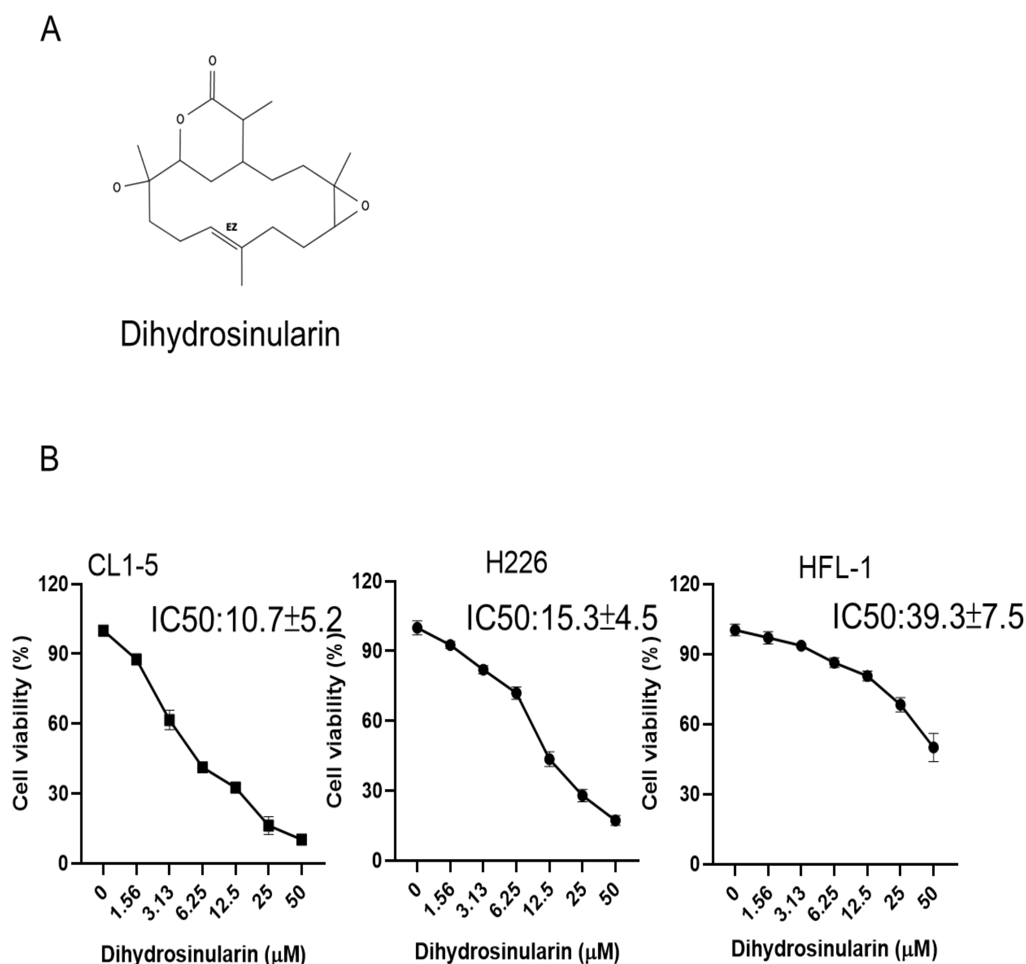

Figure 1. The effect of dihydrosinularin on the cell viability of CL1-5, H226 NSCLC, and HFL-1 normal human lung fibroblast cells. (A) The chemical structure of dihydrosinularin. (B)MTT test of CL1-5, H226, and HFL-1 cells treated with different doses of dihydrosinularin. The control (0.1 % DMSO) group had 100 % viable cells. The data are provided as the mean standard deviation (n=3).

Dihydrosinularin promotes caspase-dependent apoptosis in CL1-5 Cells

Using the Annexin V/FITC test, apoptotic cells were identified. In CL1-5 cells, a high number of viable cells was detected in the control group (0.1% DMSO) (Figure 2A and 2B). In addition, a pattern of the cell population transitioning from viable to apoptotic (annexin V+) phases was found, and this was connected with the dihydrosinularin dosage. Furthermore, flow cytometry and western blot analysis were also used to determine the activation of caspases (3, 9, and 8) and their executive substrate, PARP. Caspase-3 (Figure 3A, B), caspase-9 (Figure 3A, B), and caspase-8 (Figure 3A, B) activity were elevated in dihydrosinularin-treated CL1-5 cells in a dose-dependent manner. Furthermore, dihydrosinularin administration of CL1-5 cells increased PARP cleavage in a dose-dependent manner (Figure 3C). These findings imply that caspase-mediated apoptosis plays a role in dihydrosinularin-induced growth suppression.

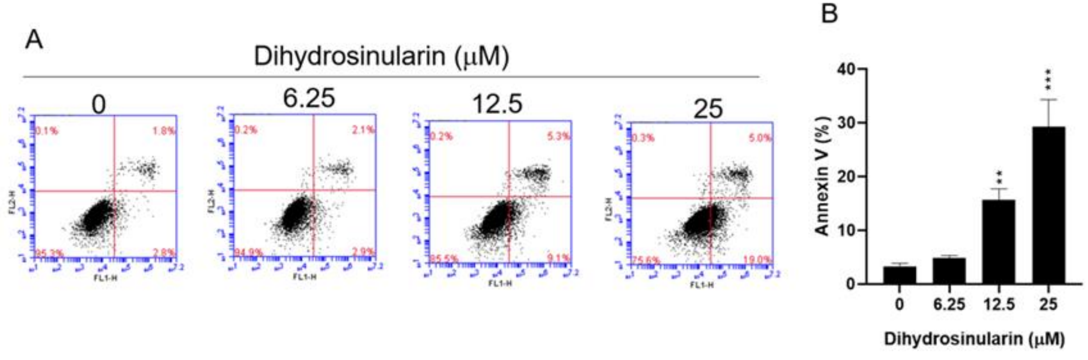

Figure 2: The effect of dihydrosinularin on CL1-5 cell apoptosis. Flow cytometry and Annexin V-FITC/PI labeling were used to examine cell apoptosis in CL1-5 cells. (A) Flow cytometry investigation of cell apoptosis. The lower right quadrants show early-stage apoptotic cells (Annexin V-FITC positive and PI negative), whereas the upper right quadrants show late-stage apoptotic cells (Annexin V-FITC and PI positive) (B) Apoptotic rate (percentage) of early and late stage apoptotic cells calculated. The data are shown as the mean standard deviation (n=3). Significant changes are noted by ** p< 0.01 and *** p< 0.001 as compared to the DMSO-treated control group.

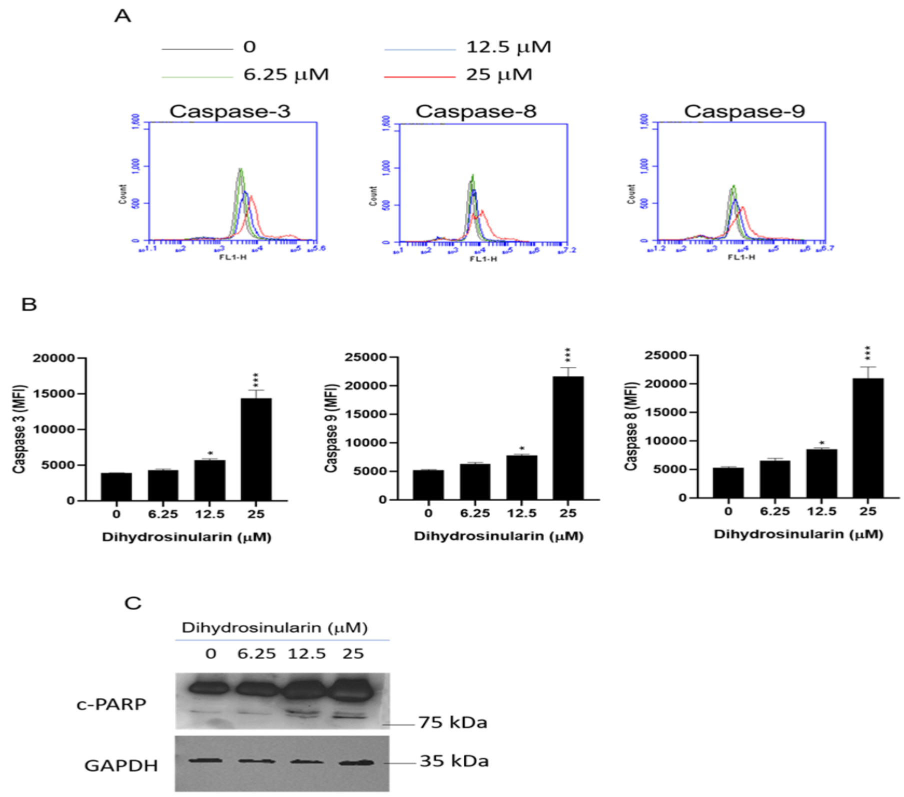

Figure 3. The effect of dihydrosinularin on caspase activity and cleaved PARP expression in CL1-5 cells. (A) histogram of caspases 3, 8, and 9 from flow cytometry of CL1-5 cancer cells treated with dihydrosinularin for 24 hours. (B) quantification of caspase 3, 98 and 9 activity in CL1-5 cells. (C) Western blot analysis for poly (ADP ribose) polymerase (PARP) cleavage was used to assess apoptosis. The data are shown as the mean standard deviation (n=3). Significant changes are noted by * p <0.05 and *** p< 0.001 as compared to the DMSO-treated control group.

Dihydrosinularin Caused Mitochondrial Dysfunction of CL1-5 cells

Mitochondria play an important role in apoptotic signaling because they connect intrinsic and extrinsic apoptotism pathways to change mitochondrial membrane permeabilization (MMP) [Lopez & Tait, 2015]. We also used JC-1 labeling to examine whether dihydrosinularin affected MMP in CL1-5 cells. As illustrated in Figures 4A and 4B, dihydrosinularin significantly reduced the MMP of CL1-5 cells in a dose-dependent manner, Furthermore, MMP is directly regulated by the Bcl-2 family of proteins during apoptosis [Levy & Claxton, 2016]. Therefore, we evaluated the expression of the proapoptotic protein Bax and the antiapoptotic protein Bcl-2 using western blotting. We observed that dihydrosinularin significantly increased Bax expression while decreasing Bcl-2 expression (Figure 4C and 4D). These results revealed that dihydrosinularin had cytotoxic effects through disrupting the membrane potential of mitochondria.

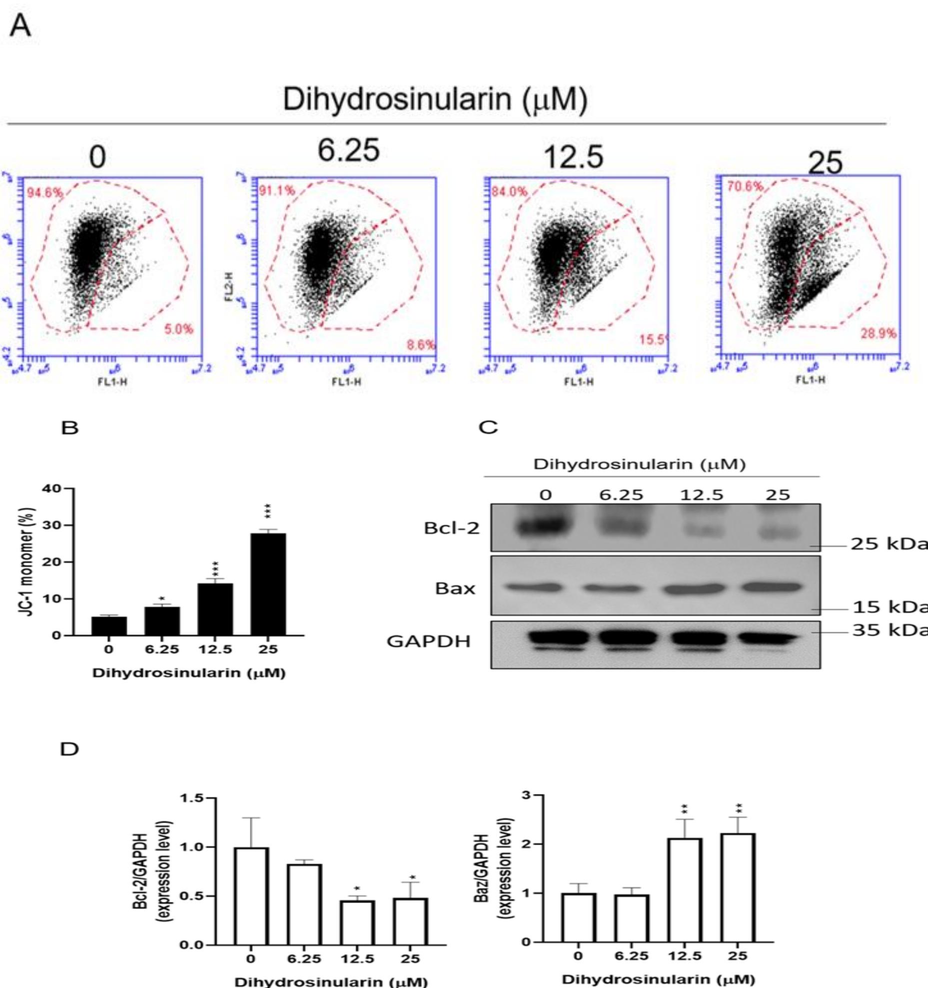

Figure 4. The effect of dihydrosinularin on CL1-5 cell mitochondrial dysfunction. (A) Using a flow cytometer, dihydrosinularin-treated cells were shown to have changed in JC1 green and red fluorescence. (B) A quantitative depiction of the proportion of JC1 (green fluorescence) data from flow cytometry. (C) Western blotting was used to evaluate the expression of Bcl-2 and Bax, two proteins linked to mitochondria. (D) Image J software's densitometric analysis demonstrating the Bax and Bcl-2 protein expression in all treatment groups. The mean and standard deviation of the data are shown (n=3). Significant changes are noted by * p <0.05, ** p< 0.01 and *** p< 0.001 as compared to the DMSO-treated control group

Dihydrosinularin Causes G2/M Phase Arrest in CL1-5 Cells

Next, we determined if dihydrosinularin promotes cell death by arresting the cell cycle. Utilizing PI staining and flow cytometry, the DNA content of the cells was determined. Figure 5A and 5B shows that treatment with 12.5 and 25 ¤M dihydrosinularin for 24 h increased the cell population in the G2/M phase compared to the control treatment, with a corresponding decrease in the cell population in the G1. Furthermore, dihydrosinularin at a concentration of 12.5 and 25 ¤M increased the number of cells in the sub-G1 phase, showing dose-dependent apoptotic cell death (Figure 5A and 5B). These findings indicate that dihydrosinularin decreased cell viability by causing G2/M cell cycle arrest and promoting apoptosis.

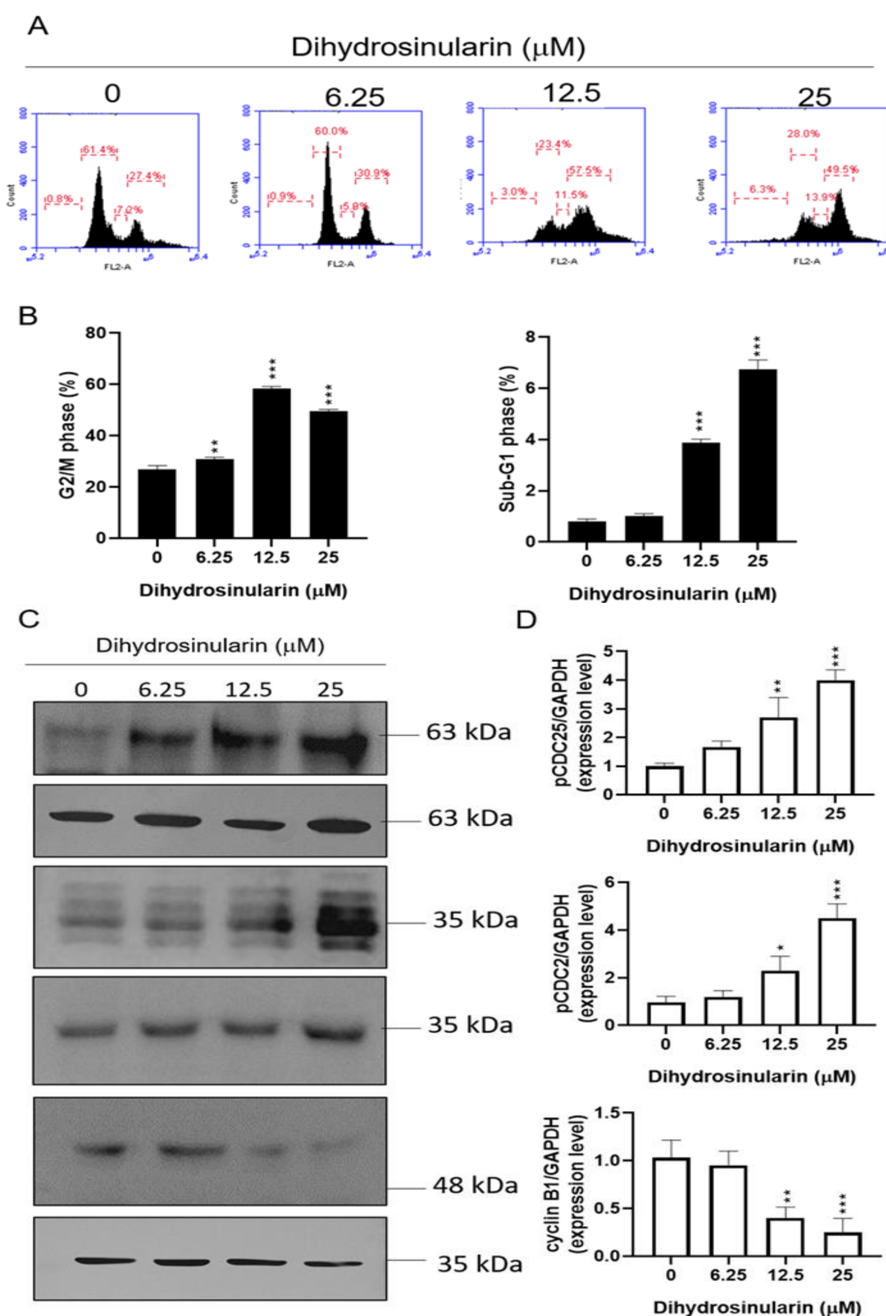

Figure 5. The effect of dihydrosinularin on the G2/M cell cycle arrest in CL1-5 cells. (A) The percentages of CL1-5 cells in various cell cycle stages following treatment with various concentrations of dihydrosinularin for 24 hours. (B) Percentages of CL1-5 cells in the G2/M and sub-G1 stages following treatment with various dihydrosinularin concentrations for 24 hours. (C) Expression of G2/M Phase-Related Proteins was evaluated through western blot. (D) Image J software densitometric analysis of the expression of G2/M Phase-Related Proteins in all treatment groups. The data are shown as the mean standard deviation (n = 3). Significant changes are noted by * p <0.05, ** p< 0.01 and ***P<0.001 as compared to the DMSO-treated control group

Dihydrosinularin affected the expression of G2/M Phase-Related Proteins in CL1-5 Cells

Next, we examined the expression of proteins associated with the G2/M phase of the cell cycle following dihydrosinularin treatment. The findings revealed that phospho-Cdc25C (Ser-216) and phospho-Cdc2 (Tyr-15) expression was enhanced, but cyclin B1 expression were dose-dependently reduced (Figure 6 A and 6B). These findings suggested that dihydrosinularin could cause G2/M arrest by changing the expression of G2/M phase-related proteins.

Dihydrosinularin Induces ATM/Chk2 Protein Phosphorylation in CL1-5 cells

p53 protein can be regulated by DNA damage-sensing kinases such as ATM, ATR, and checkpoint kinases (Chk1 and Chk2) [Banin *et al.*, 1998; Hirao *et al.*, 2000]. The ATM/ATR pathway is a crucial regulatory point in DNA homologous recombination repair. To determine whether dihydrosinularin activates DNA

damage-sensing kinases, the phosphorylation of these kinases was analyzed by western blotting. As shown in Figure 6A and 6B, dihydrosinularin treatment significantly raised the expression of p-H2A.X, p-ATM (Ser1981) and p-Chk2 (Ser19) at 24 hours, but had no effect on the expression of p-ATR (Ser428) or p-Chk1 (Ser317). These results imply that dihydrosinularin may induce DNA damage via ATM /chk2 signaling pathway.

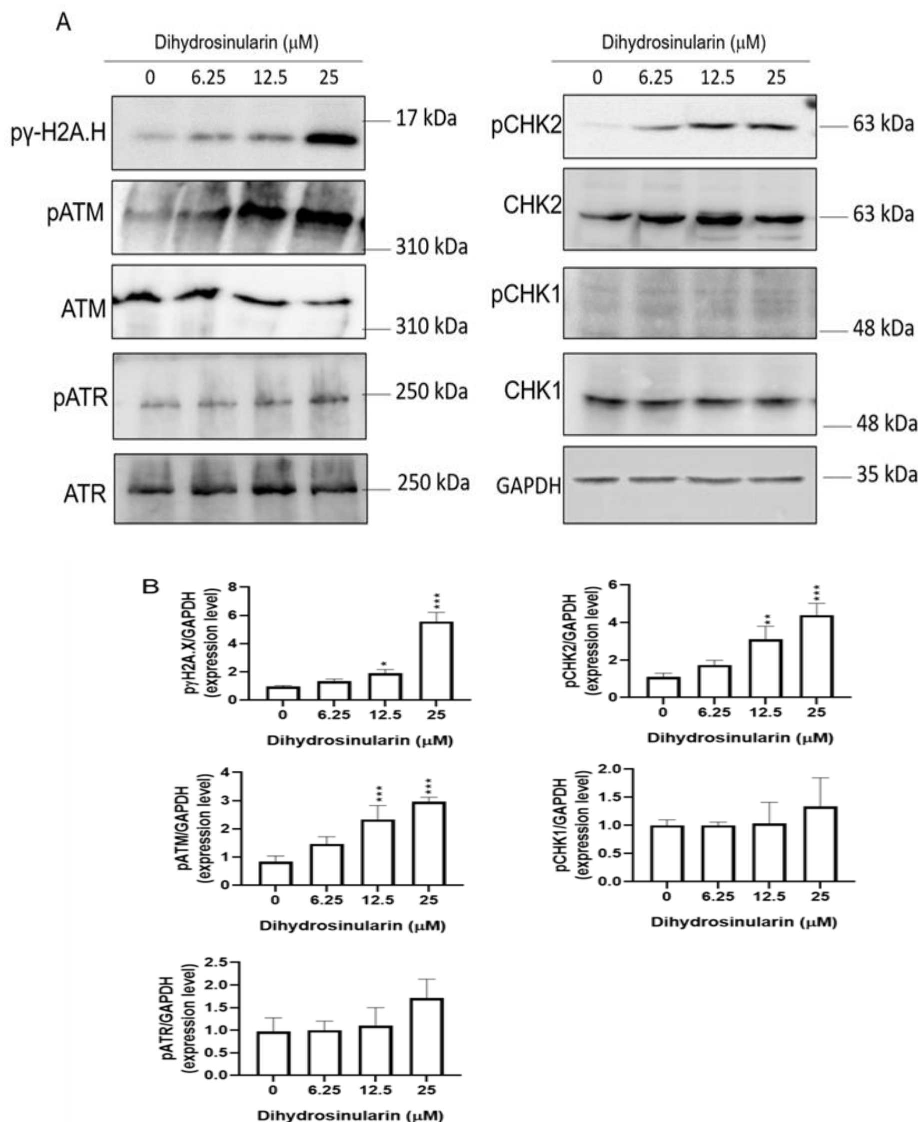

Figure 6. The effect of dihydrosinularin Induces ATM/Chk2 protein phosphorylation in CL1-5 cells. CL1-5 cells were treated with various dihydrosinularin concentrations for 12 hours. (A) Western blotting was used to evaluate the expression of DNA damage-related proteins. (B) Densitometric study of the expression of DNA damage-related proteins in all treatment groups utilizing Image J software. The data are shown as the mean standard deviation (n = 3). Significant changes are noted by * p <0.05, ** p< 0.01 and ***P<0.001 as compared to the DMSO-treated control group

DISCUSSION

Lung cancer is the major cause of cancer-related death, accounting for approximately 25% of all cancer-related deaths [Siegel *et al.,* 2021]. For decades, chemotherapy has been the primary treatment for NSCLC. [Li *et al.,* 2019] However, medication resistance is the most significant factor limiting the effectiveness of chemotherapy. Consequently, the development of innovative chemotherapeutic drugs is essential.

Recent reports indicate that dihydrosinularin inhibits cell growth in numerous tumors. In this work, the anticancer effects of dihydrosinularin were examined in human NSCLC cell lines. Our data reveal for the first time that dihydrosinularin induces G2/M arrest and apoptosis in NSCLC. Additionally, DNA damage may have contributed to the dihydrosinularin-induced cell death. These phenomena are described graphically in Figure 7.

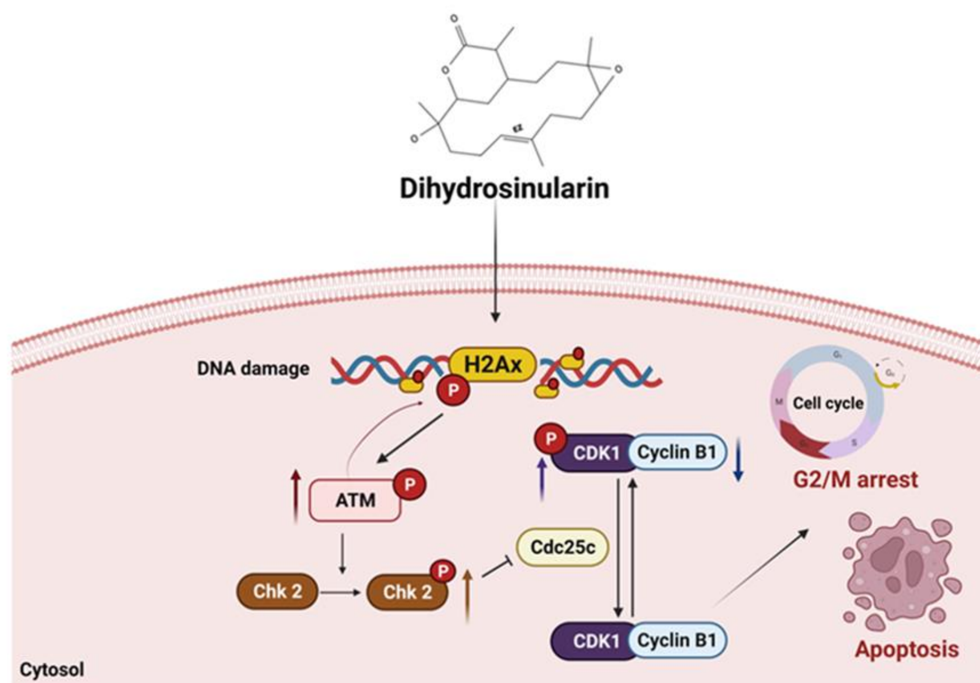

Figure 7. Our proposed model of the mechanism underlying the antitumor effects of dihydrosinularin on NSCLC cells. Dihydrosinularin induces G2/M arrest and apoptosis in human CL1-5 NSCLC cells via DNA damage-mediated ATM/CHK2 kinase signaling cascades.

Most chemotherapies induce apoptosis, an irreversible form of programmed cell death mediated by two different mechanisms: the intrinsic and extrinsic pathways [Bai & Wang , 2014]. The intrinsic mechanism, commonly known as mitochondrial apoptosis, is regulated by pro- and antiapoptotic Bcl-2 family proteins [Shiloh & Ziv, 2013]. The extrinsic route induces apoptosis by activating the caspase-8 initiator. Both routes converge at caspase-3, together with alterations in other effectors important for carrying out apoptosis [Sahoo *et al.*, 2023]. Previous research has demonstrated that dihydrosinularin can induce apoptosis via the caspase pathway [Yang *et al.*, 2021], but it is unclear whether this occurs via the intrinsic or extrinsic mechanism. In this study, we noticed that dihydrosinularin increased expression of proapoptotic Bax and decreased expression of antiapoptotic Bcl-2 (Fig. 4C), which, in conjunction with increasing mitochondrial permeability (Fig. 4A and 4B), activated caspase-9 (Fig 3A and 3B). Moreover, our flow cytometry data (Fig. 3A and 3B) revealed that dihydrosinularin can boost caspase-8 activation. These findings suggested that dihydrosinularin can activate both the intrinsic and extrinsic pathways of apoptosis in lung cancer cells. However, the mechanisms that initiate these apoptosis pathways require further investigation.

The most prevalent mechanism mediated by conventional chemotherapeutic medicines is cell cycle arrest [Otto & Sicinski, 2017]. In the G2/M transition, the protein complex M phase-promoting factor, composed of cdc2 and Cyclin B1, is crucial. In humans, cdc25A, cdc25B, and cdc25C dephosphorylate cdc2 to promote the activity of this complex. Specifically, cdc25C dephosphorylates cdc2/Cyclin B1 in order to overcome the G2/M checkpoint and enter mitosis, and their down-regulation results in G2/M cell cycle arrest [Liu *et al*,. 2019]. Unlike previous studies [Yang *et al.*, 2021], we discovered for the first time that dihydrosinularin can induce G2/M arrest in NSCLC cells in a dose or time-dependent manner (Fig 4A), and immunoblot showed that dihydrosinularin increased phophos-cdc25C (ser 216) and phosphor cdc2 (tyr 15), while decreasing cyclin B1 at the protein level in CL1-5 cells (Fig 4C). These results show that the G2/M arrest associated with dihydrosinularin may entail phosphorylation of cdc25C and cdc2 as well as downregulation of cyclin B1.

In addition, ATM is essential for the activation of cell cycle checkpoints [Lavin, 2019]. ATM is promptly and selectively activated in response to this activation as well as other forms of cellular stress [Bakkenist & Kastan, 2003; Gibson *et al.*, 2005]. Activated ATM can modulate the phosphorylation status and, consequently, the activity of Chk2, which triggers G2/M cell cycle arrest by increasing the phosphorylation of cdc25c [Sancar *et al.*, 2004]. Although prior research has demonstrated that dihydrosinularin produces DNA damage, the mechanism remains unknown [Yang *et al.*, 2021]. In the present investigation, we discovered that dihydrosinularin activated ATM and Chk2, indicating that the mechanisms responsible for dihydrosinularin's effects on G2/M phase arrest may be related to the control of the ATM-Chk2 signaling pathway. To verify the detailed mechanism, however, additional experimental are necessary.

Some ROS-modulating drugs have been developed for anticancer therapy with antiproliferation properties [Lee *et al.*, 2013; Widodo et al., 2010]. Dihydrosinularin is a possible ROS-modulating agent because it contains antioxidant properties [Wang *et al.*, 2021]. However, earlier research has shown that dihydrosinularin can trigger apoptosis and DNA damage in oral cancer cells by producing reactive oxygen species [Yang *et al.*, 2021]. However, in our present investigation, we observed that dihydrosinularin treatment reduced the expression of endogenous ROS in NSCLC cells (data not shown). As a result, the association between dihydrosinularin and ROS in various cancer types has to be studied and defined further.

According to the results of previous studies of drug cancer cell cytotoxicity, in triple-negative breast MDA-MB-231, lung H1299

cells, and liver HA22T/VGH cancer cells, the IC50 values of dihydrosinularin at 24h in MDA-MB-231, H1299, and HA22T/VGH cells were 60, 70, and 120 μM, respectively [Wang *et al.*, 2021]. In oral cancer cells (Ca9-22, OECM-1, CAL 27, and SCC-9), the IC50 values of dihydrosinularin at 48 h were: 0.39, 0.69, 0.8 and 0.65 mM, respectively [Yang *et al.*, 2021]. However, in our present, we found that the IC50 values of dihydrosinularin at 24 h were 10.7± 5.2 μM and 15.3 ± 4.5 μM in CL1-5 and H226 cells, respectively. These result suggests that dihydrosinularin has quite different sensitivities to the cytotoxicity of different cancer cells, but the mechanism for the difference remains unclear. Therefore, to further test the antitumor effect of dihydrosinularin on more different cancer cell lines, and to understand more detailed mechanisms of action, is very important work for the application of dihydrosinularin to future drug development.

In summary, the anti-proliferative effect of dihydrosinularin and its underlying mechanism were studied in the CL1-5 NSCLC cell line. Dihydrosinularin induces G2/M cell cycle arrest and apoptosis through ATM/Chk2 signaling pathways, associating with induction of DNA damage, which offer a potential anti-tumor agent against NSCLC cancer. Further studies will be performed in vivo to confirm these effects of dihydrosinularin.

Ethical approval

This study did not involve human participants. All experimental procedures followed standard protocols and were conducted in accordance with the ethical standards and regulations of the laboratory. The use of cell lines complied with the ethical standards and norms of the laboratory.

Funding details

This work was partly supported by Grant TTMHH-R1110078 from Tungs' Taichung MetroHarbor Hospital and Changhua Christian Hospital (111-CCH-IRP-029).

Conflict of interest

The authors declare that they have no conflict of interest.

Informed Consent

Informed consent was obtained from all individual participants included in the study.

Authorship contributions

CYL and YHC devised the research project. CYL, YHC, and YXC conducted the experiments and analyzed the data. CYL, YHC, and TCY aided in the animal experiments. YJL, KWC, and SHL drafted the manuscript. KWC and SHL oversaw the experiments and provided a critical review of the manuscript. All authors approved the final version of the manuscript.

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