Cimigenoside Affects Cell Viability, Apoptosis and Metastasis of A549 Cells via the NF-kB Pathway

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Accepted June 23, 2022 Published online June 30, 2022 Issue online June 30, 2022 Original article WANG Q., YANG H., YANG W., JIANG T. 2022. Cimigenoside affects cell viability, apoptosis and metastasis of A549 cells via the NF-KB pathway. Folia Biologica (Kraków) 70: 85-91. Cimigenoside, one of the main elements isolated from the aboveground plants of the Cimicifuga genus, has anti-tumour effects. However, the question of whether Cimigenoside had effects on lung cancer remains elusive. This study was aimed at investigating the effects of Cimigenoside on A549 lung cancer cells. The A549 cells were cultured in vitro, and an MTT assay was carried out to explore the cell proliferative abilities after a treatment with Cimigenoside. The wound healing assays and Transwell invasion assays were carried out to explore the effects of Cimigenoside on cell migration and invasion in the A549 cells. A cell apoptosis assay using flow cytometry was used to evaluate the effects on cell apoptosis in the A549 cells. The related protein expression levels were measured using a Western blot assay. It was hypothesised that Cimigenoside could restrain tumour cell proliferation, migration and invasion, while inducing cell apoptosis with apoptosis-related protein change levels in a time- and dose-dependent manner. The Western blot assay revealed that the Cimigenoside treated A549 cells had a reduced expression of p65, while having an increased expression of $I\kappa B\alpha$ at the protein level. Taken together, this study demonstrated that Cimigenoside has the potential to repress cell proliferation, migration and invasion, while inducing apoptosis in A549 lung cancer cells through the NF-kB pathway. These results reveal that Cimigenoside may have a therapeutic potential for lung cancer treatment. Key words: Lung cancer, proliferation, migration, invasion, mechanism. Qinqin WANG, Department of Pulmonary and Critical Care Medicine, Yantai Yuhuangding Hospital, Yantai, 264099, Shandong, P.R, China. Hua YANG, Department of Pulmonary and Critical Care medicine, Yantai Hospital of traditional Chinese Medicine, Yantai, 264013, Shandong, P.R, China. Wenjun YANG[⊠], Department of Neurology, Yantai Yuhuangding Hospital, 20 Yuhuangding East Road, Zhifu District, Yantai, 264099, Shandong, P.R, China. E-mail: yang5xun@163.com Tingshu JIANG^{$\square}$, Department of Pulmonary and Critical Care Medicine, Yantai Yuhuangding Hospi-</sup> tal, 20 Yuhuangding East Road, Zhifu District, Yantai, 264099, Shandong, P.R, China. E-mail: Tingshuvt@163.com ^a These authors contributed to the study equally.

Lung cancer results in malignant tumours that present a seriously threat to human health and life in the world today, with the morbidity and mortality ranking first among malignant tumour diseases (SIEGEL & MILLER 2020). Histologically, the types of lung cancer include small cell lung cancer (SCLC) and nonsmall cell lung cancer (squamous cell carcinoma, adenocarcinoma, large cell carcinoma and branchial glandular carcinoma) (SINGH & CHELLAPPAN 2014). At present, the clinical treatment of lung cancer is still a comprehensive form of treatment based on chemotherapy. Although the commonly-used chemotherapeutics administered in a clinic have definite anti-tumour effects and a remarkable curative efficacy, the accompanying bone marrow suppression, gastrointestinal reactions side effects and drug resistance seriously af-

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fect the tolerance and therapeutic effects for patients with lung cancer (CAO *et al.* 2019). Nowadays, Chinese medicine therapy occupies a place of great importance in disease therapy, as it has lesser side effects and toxicity; thus, a number of studies have been dedicated to identifying neo-drugs with the possibility for lung cancer treatment (QIU *et al.* 2019). Therefore, searching for and discovering new natural compounds with anti-tumour effects will contribute to tumour prevention and treatment, as well as improving the quality of life for patients and contributing to better human health.

Cimicifuga is a perennial herb with abundant resources, which is widely distributed and has various varieties in China (Y. GUO et al. 2017). The chemical composition of cohosh is complex, containing more than 200 compounds that mainly include triterpenoid saponins, phenolic acids and volatile oils. These compounds have anti-tumour, analgesic and anti-inflammatory, anti-viral, immunomodulatory components and other pharmacological effects (HUYEN & LUYEN 2018). Cimigenoside (also called Cimigenol 3-β-D-xyloside / Cimigenol xyloside / Cimigoside) is a cyclic jackfruit triterpenoid isolated from the aerial parts of the genus Cimicifuga dahurica (JIA et al. 2021; TIAN et al. 2005). Several studies have demonstrated that the triterpenoids isolated and identified from cohosh plants can induce apoptosis in some tumour cell lines in vitro (LI et al. 2020; SALVADOR et al. 2017; WANG et al. 2014). For instance, 23-O-acetylcimigenol-3-O-β-D-xylopyranoside could inhibit the proliferation of HepG2 cells and induce cell apoptosis HepG2 cells (TIAN et al. 2006). However, determining whether Cimigenoside has anti-tumour effects in the case of lung cancer remains elusive.

The nuclear factor- κB (NF- κB) transcription factor family plays a crucial role in the progression of lung cancer, affecting viabilities, migration and apoptosis of cancer cells (PERKINS 2012; RASMI et al. 2020). There is evidence for pharmacological effects of triterpenoids that are associated with multiple signalling pathways, including the NF-κB pathway (XU et al. 2018; ZHANG et al. 2020). A previous study reported that a cycloartane triterpenoid KHF16 (24-acetylisodahurinol-3-O-β-D-xylopyranoside) isolated from Cimicifuga foetida could suppress the progression of breast cancer by inhibiting the NF-kB signalling pathway in part (KONG et al. 2016). Therefore, we proposed the hypothesis that Cimigenoside might exert anti-tumour effects on lung cancer cells by inhibiting the NF- κ B activities.

In the current study, the effects of Cimigenoside on the proliferation and apoptosis of human lung cancer A549 cells were observed. Moreover, the possible mechanism of action was explored, in order to provide an experimental basis for its clinical application.

Materials and Methods

Cell culture

A human lung cancer cell line A549 was purchased from the American Type Culture Collection (ATCC; USA) and maintained in Dulbecco's modified Eagle's medium (DMEM; Giboco, Carlsbad, CA, USA), supplemented with a 10% foetal bovine serum (FBS; Gibco) in the presence of 100 IU/ml penicillin and 100 μ g/l streptomycins. The cells were incubated in a humidified atmosphere with 5% CO₂ air at 37°C.

Reagents

Cimigenoside ($C_{35}H_{56}O_9$, purity $\ge 98\%$, relative molecular mass = 620.83) was purchased from Sigma-Aldrich Trading Co., Ltd (Shanghai, China). The chemical structure of the Cimigenoside is shown in Figure 1A. The Cimigenoside was dissolved in 0.1% DMSO as a stock solution of 10 mM. Then, the cells were treated with different concentrations (1, 2, 5 µmol/l) of Cimigenoside. The cells were used in cell viability, metastasis, invasion and apoptosis assays.

Cell viability assay

The effect of Cimigenoside's cytotoxicity on A549 cells was investigated using a 3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay. There were three assays repeated for one group, and the A549 cells (20,000 cells/well) were seeded in 96-well plates with medium-added drugs. In the control group, the same volume of culture medium was added with 0.1% DMSO. After an incubation of 48 h, the MTT reagent was added to each well and it was further incubated at 37°C for 1 h. The absorbance at 570 nm was measured using a DTX 880 Multimode Detector (Backman Coulter, Brea, CA, USA).

Cell scratch test assay

The effect of Cimigenoside on the A549 cells was analysed using Cell scratch test assays. The cells were resuspended in a serum-free medium containing Cimigenoside and plated in 6-well plates. The cell sheets were wounded with a plastic pipette tip. After an incubation of 24 h, the wound gap width was photographed using a Nikon TE300 microscope and was quantified using Image-Pro Plus software (Media Cybernetics, MD, USA).

Transwell invasion assay

The cell invasion abilities of the A549 cells after treatment with Cimigenoside were detected by a Matrigel invasion assay (8 µm pore size; BD Biosci-



Fig. 1. The effects of Cimigenoside on cell proliferation and apoptosis in A549 cells. A. Chemical structure of Cimigenoside. B. The Cimigenoside suppressed cell proliferation in a time- and dose-dependent manner. The data was compared with the control using a two-way ANOVA. C. The Cimigenoside induced cell apoptosis in a dose-dependent manner. The data was compared with the control using a one-way ANOVA. D. The apoptosis-related protein levels were detected in the A549 cells using a Western blot assay. The data was compared with the control using a two-way ANOVA. * p<0.05, ** p<0.01, *** p<0.001 compared to the control.

ences, USA). The cells $(1 \times 10^5$ cells) in a serum-free DMEM medium were added to the upper chamber, while a complete medium with 10% FBS was put into the bottom chamber. After 24 h of incubation, the invaded cells were fixed with 4% polyformaldehyde, stained by 0.1% crystal violet, and the cell numbers were counted under a light microscope (Leica).

Cell apoptosis assay

The induction of cell apoptosis in the A549 cells by Cimigenoside was quantified by an Annexin V-FITC Apoptosis Detection Kit (Sigma, Billerica, MA, USA). After treatment with Cimigenoside, the cells were incubated with annexin V and PI in the dark for 15 min, then the cell apoptosis was assessed by flow cytometry (BD Bioscience).

Western blot assay

The A549 cells were washed by PBS, then lysed in an RIPA lysis buffer (Thermo Fisher Scientific, USA) with protease inhibitors to isolate the proteins. The protein concentration was measured using the BCA protein kit (Beyotime, Shanghai, China). The protein (15 μ g) was separated in 10% polyacrylamide gels for SDS-PAGE and was transferred onto a PVDF membrane (Millipore, Bedford, MA, USA). Subsequently, the PVDF membrane was blocked in 5% non-fat milk overnight at 4°C, and was then incubated with primary antibodies (Abcam; USA) at room temperature for 3 h. After that, the blots were incubated for 1 h with secondary antibodies (Abcam) tagged with horseradish peroxidase (HRP) at room temperature. The protein grey blots on the PVDF membrane were visualised with an enhanced chemiluminescence (ECL) kit (Millipore) and were recorded with ImageJ and normalised to GAPDH.

Statistical analysis

All the statistical analyses were carried out using GraphPad Prism 7.0 software and the data was presented as the mean \pm SD. The statistical significance among the groups was analysed using a one-way ANOVA or two-way ANOVA, followed by a Tukey post hoc test. A p-value lower than 0.05 was considered to indicate a statistically significant difference.

Results

Cimigenoside suppresses the proliferation of lung cancer A549 cells

The cell cytotoxicity of Cimigenoside was examined using an MTT assay. The A549 cells were exposed to 0, 1, 2 and 5 μ mol/l of Cimigenoside for 0, 24, 48 and 72 h. The results in Figure 1B show that Cimigenoside suppressed the viabilities of the A549 cells in a time- and dose-dependent manner (p<0.05).

Cimigenoside induces cell apoptosis in A549 cells

To explore the effect of Cimigenoside on lung cancer cell apoptosis, the A549 cells were cultured with different concentrations of Cimigenoside for 48 h. It was observed that Cimigenoside promoted A549 cell apoptosis in a dose-dependent manner (p<0.01, Figure 1C).

Furthermore, the apoptosis-related proteins were also measured using a Western blot assay after treatments with different doses of Cimigenoside for 24 h. The results shown in Figure 1D revealed that Cimigenoside decreased the expression of anti-apoptotic Bcl-2 and accelerated the expression of pro-apoptotic proteins Bax, caspase-3 and caspase-9 (p<0.01). Only the 1 μ mol/l of Cimigenoside showed a changing trend, but it had no significant statistical difference. These results suggest that Cimigenoside could promote apoptosis in lung cancer cells in a dose-dependent manner.

Cimigenoside inhibits lung cancer cell migration and invasion

Additionally, the effects of Cimigenoside on cell migrative and the invasive capacities were investigated using a cell scratch test assay and a Transwell invasion assay, respectively. The results exhibited in Figure 2A reveal that Cimigenoside inhibited the cell migration with small, closed wound areas (wider scratches) in a dose-dependent manner (p<0.05). The Transwell invasion assay results indicated that Cimigenoside caused less cells to invade through the membrane in a dose-dependent manner, compared with the control (p<0.01, Figure 2B). In summary, the results revealed that Cimigenoside could weaken the migration and invasive capacity of cancer cells in a dose-dependent manner.

Cimigenoside interferes with the activation of NF- κ B signalling in lung cancer cells

The Western blot assay revealed that the Cimigenoside treated A549 cells had a reduced expression of p65, while having an increased expression of I κ B α at the protein level (p<0.01, Figure 3). These results illustrated that Cimigenoside can significantly suppress the cell viabilities and invasive abilities, while inducing apoptosis through a negative modulation of the NF- κ B pathway in A549 cells.

Discussion

Lung cancer, as a main cause of malignant tumours, has a high morbidity and mortality rate and its treatment has become a global burden. The current existing therapies may cure lung cancer to some extent, but the five-year overall survival rate remains at about 20% on account of metastasis, chemoresistance and recurrence (SIEGEL & MILLER 2022). Traditional Chinese medicine is regarded as a novel potential therapeutic strategy with a higher level of tolerance. Cimigenoside is one of the main active cyclic jackfruit triterpenoid compounds. Previous studies have demonstrated that triterpenoid induces cancer cell apoptosis in some cancer cell lines (JIA et al. 2021), but whether Cimigenoside induces apoptosis in lung cancer cells has remained elusive. In the current study, we verified that Cimigenoside can promote cell apoptosis and suppress cell proliferation in vitro, which further illustrated the possible mechanism leading to cell death.

Triterpenoids, as metabolites of isopentenyl pyrophosphate oligomers, are used in many clinics in Asian countries (PETRONELLI *et al.* 2009). Previous studies have demonstrated that triterpenoids possess chemotherapeutic effects in cell lines and animal models, as well as in clinical trials (BISHAYEE *et al.* 2011; LI *et al.* 2020). Triterpenoids can prevent cancers through the biological properties of epigenetic/epigenomic regulation, antioxidant and anti-inflammatory activities (OVESNÁ *et al.* 2004). An increasing number of studies have reported that triterpenoids have no toxicity in normal cells, but exhibit cytotoxicity against numerous cancer cells



Fig. 2. The effects of Cimigenoside on cell migration and invasion in A549 cells. A. A cell scratch test assay was used to measure the cell migration abilities. The data was compared with the control using one-way ANOVA. * p<0.05, ** p<0.01, *** p<0.001. B. A Transwell invasion assay was carried out to detect the invasive capacities. Line – mean; box – 25^{th} through 75^{th} percentiles; whiskers – min to max show all points; points – raw data. The comparison was carried out using a one-way ANOVA. * p<0.05, ** p<0.01, *** p<0.001, ***



Fig. 3. Expression of the proteins associated with the NF- κ B signalling following various treatment concentrations of A549 cells with Cimigenoside. The expression of p65, p-p65, I κ B α , p-I κ B α , and GAPDH was analysed by a Western blot assay and compared using a two-way ANOVA. ** p<0.01, *** p<0.001 compared with the control.

(QIAN et al. 2020). For instance, one of the synthetic triterpenoids, 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO) methyl ester, has anti-tumour effects via the inhibition of tumour cell growth and a contribution to apoptosis in several solid cancers, such as pancreatic cancer (DEEB et al. 2012), colorectal cancer (GAO et al. 2010), ovarian cancer (GAO et al. 2013), breast cancer (ZHOU et al. 2020) and prostate cancer (KHURANA & CHANDRA 2020). The present study shows that the proliferation rates of A549 cells were restrained in a time- and dose-dependent manner. The effect of Cimigenoside on cell migration and invasive abilities was also explored. The results demonstrated that the healing rates and invasive abilities were weakened with increasing drug concentrations. In addition, the apoptosis rates of A549 cells increased when the concentrations of Cimigenoside were higher. These results were consistent with the reported effects of Cimigenoside on proliferation and metastasis in breast cancer cells (JIA et al. 2021). Another compound from Cimicifuga, 23-O-acetylcimigenol-3-O-β-D-xylopyranoside, exerted its cytotoxicity via an inhibition of the proliferation and induction of apoptosis in hepatocellular carcinoma cells (HepG2) (TIAN et al. 2006). A study in relation to breast cancer also reported that cycloartane triterpenoids (25-O-acetylcimigenol-3-O-β-D-xylopyranoside, 25-O-acetylcimigenol-3-O-α-L-arabinopyranoside and 23-O-acetylcimigenol-3-O-β-D-xylopyranoside) from Cimicifuga yunnanensis could induce the cancer cell apoptosis of MCF7 cells through the p53-dependent mitochondrial pathway (FANG et al. 2011).

Mechanistically, Cimigenoside suppresses the activation of NF-kB signalling in A549 cells. NF-kB is a crucial transcription factor that plays a crucial role in cell growth, progression and the resistance of human cancers (PRAMANIK & MAKENA 2018. Numerous studies have demonstrated that Chinese medicine could mediate the progression of tumours through the NF-κB pathways (Z. GUO et al. 2020; JIAO et al. 2019; SONG et al. 2020). To further illuminate the potential mechanisms involved in the effects of Cimigenoside-induced apoptosis on lung cancer, a Western blot assay was used to evaluate the expression levels of some proteins in the NF-κB pathway of A549 cells. In this study, it was observed that an increased concentration of Cimigenoside decreased the expression of p65, while increasing the expression of IκBα at the protein level, which revealed that Cimigenoside could inhibit the activation of NF-kB signalling in a dose-dependent manner. These results support the hypothesis that the induction of apoptosis and the suppression of the proliferation, migration and invasion in cells by Cimigenoside is mediated by a suppression of the NF- κ B pathway. However, the lack of *in vivo* experiments is a limitation in this study. In future studies, the results acquired from the *in vitro* study and the molecular mechanism of Cimigenoside need to be verified through in vivo experiments.

Taken together, this study demonstrated that Cimigenoside has the potential to repress cell proliferation, migration and invasive abilities, while inducing apoptosis in A549 lung cancer cells through the NF- κ B pathway. These results reveal that Cimigenoside may have a therapeutic potential for lung cancer treatment. The present study results provide a basis for the clinical treatment of lung cancer, and the anti-tumour functions of Cimigenoside will be investigated in clinical trials.

Author Contributions

Research concept and design: Q.W., W.Y., T.J.; Collection and/or assembly of data: H.Y.; Data analysis and interpretation: Q.W., H.Y.; Writing the article: Q.W.; Critical revision of the article: H.Y., W.Y., T.J.; Final approval of article: Q.W., H.Y., W.Y., T.J.

Conflict of Interest

The authors declare no conflict of interest.

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