

Inactivation of AKT/NF- κ B signaling by eurycomalactone decreases human NSCLC cell viability and improves the chemosensitivity to cisplatin

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Received February 26, 2020; Accepted July 3, 2020

DOI: 10.3892/or.2020.7710

Abstract. The high activation of protein kinase B (AKT)/nuclear factor- κ B (NF- κ B) signaling has often been associated with the induction of non-small cell lung cancer (NSCLC) cell survival and resistance to cisplatin, which is one of the most widely used chemotherapeutic drugs in the treatment of NSCLC. The inhibition of AKT/NF- κ B can potentially be used as a molecular target for cancer therapy. Eurycomalactone (ECL), a quassinoid from *Eurycoma longifolia* Jack, has previously been revealed to exhibit strong cytotoxic activity against the human NSCLC A549 cell line, and can inhibit NF- κ B activity in TNF- α -activated 293 cells stably transfected with an NF- κ B luciferase reporter. The present study was the first to investigate whether ECL inhibits the activation of AKT/NF- κ B signaling, induces apoptosis and enhances chemosensitivity to cisplatin in human NSCLC cells. The anticancer activity of ECL was evaluated in two NSCLC cell lines, A549 and Calu-1. ECL decreased the viability and colony formation ability of both cell lines by inducing cell cycle arrest and apoptosis through the activation of pro-apoptotic caspase-3 and poly (ADP-ribose) polymerase, as well as the reduction of anti-apoptotic proteins Bcl-xL and survivin. In addition, ECL treatment suppressed the levels of AKT (phospho Ser473) and NF- κ B (phospho Ser536).

Notably, ECL significantly enhanced cisplatin sensitivity in both assessed NSCLC cell lines. The combination treatment of cisplatin and ECL promoted cell apoptosis more effectively than cisplatin alone, as revealed by the increased cleaved caspase-3, but decreased Bcl-xL and survivin levels. Exposure to cisplatin alone induced the levels of phosphorylated-AKT and phosphorylated-NF- κ B, whereas co-treatment with ECL inhibited the cisplatin-induced phosphorylation of AKT and NF- κ B, leading to an increased sensitization effect on cisplatin-induced apoptosis. In conclusion, ECL exhibited an anticancer effect and sensitized NSCLC cells to cisplatin through the inactivation of AKT/NF- κ B signaling. This finding provides a rationale for the combined use of chemotherapy drugs with ECL to improve their efficacy in NSCLC treatment.

Introduction

Lung cancer is the most common type of cancer, in terms of both occurrence and mortality. Non-small cell lung cancer (NSCLC), which includes adenocarcinoma, squamous cell carcinoma and large cell carcinoma, accounts for ~85% of all lung cancer cases and is associated with a poor prognosis (1). The principle chemotherapeutic agent for patients with NSCLC is platinum-based compounds, especially cisplatin. However, the overall 5-year survival rate of NSCLC treatment with platinum-based regimens remains low at 16% (2). Platinum-based regimens also appear to have a higher toxicity compared with non-platinum-based regimens (3). Therefore, it would be beneficial to identify novel anticancer agents that can improve the efficacy and reduce the toxicity of platinum-based chemotherapy for NSCLC treatment.

The protein kinase B (AKT)/nuclear factor- κ B (NF- κ B) signaling pathway is vital for cell growth, survival and apoptosis (4). The serine/threonine-protein kinase AKT is activated via specific phosphorylation at Thr-308 or Ser-473 by phosphatidylinositol-3-kinase (PI3K) (5), and the activated AKT can subsequently regulate the activation of NF- κ B to control the expression of cell survival regulators (6,7). The AKT/NF- κ B signaling pathway is constitutively activated in several types

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Key words: eurycomalactone, cisplatin, chemosensitivity, non-small cell lung cancer, protein kinase B, nuclear factor κ B

of cancer, including NSCLC (6). Furthermore, the high AKT/NF- κ B activation has also been demonstrated as a key mechanism of acquired cisplatin resistance by increasing the threshold for cell death induction (8,9). Exposure to cisplatin activates AKT/NF- κ B signaling, which consequently induces the expression of NF- κ B target genes, including anti-apoptotic genes and genes involved in cell survival, such as Bcl-xL and survivin. These events lead to the inhibition of cisplatin-induced apoptosis and subsequently the development of cisplatin resistance (10,11). Currently, there is an increasing worldwide interest in plant phytochemicals from a health perspective, due to epidemiological and clinical studies reporting the benefits of plant consumption in lowering the risk of cancer (12). The inactivation of AKT/NF- κ B signaling by phytochemicals, such as genistein (13,14), baicalein (15) and tunicamycin (16), has been revealed to contribute to a reduction in cancer cell viability and tumor progression and enhance the efficacy of therapeutic cisplatin in NSCLC *in vitro* and *in vivo*. Targeting the AKT/NF- κ B pathway using plant chemicals is a promising approach for enhancing cisplatin sensitivity in NSCLC.

The present study focused on eurycomalactone (ECL; Fig. 1A), an active natural C-19 quassinoid compound isolated from *Eurycoma longifolia* Jack, a popular herbal medicine used in Southeast Asian countries (17). Its root and rhizome extract have been traditionally used to treat various conditions and diseases, including sexual dysfunction, malaria, diabetes, anxiety, aches, fever, constipation and cancer (18). The *in vitro* preliminary screening for the anticancer potential of several quassinoids, identified the main bioactive compounds derived from *E. longifolia*. Notably, among the isolated quassinoids, ECL has displayed the most potent anticancer effect against various cancer cell lines, including human NSCLC A549 cell line (19,20). However, the mechanisms underlying the strong cytotoxicity of ECL against human NSCLC cells have not been investigated. Our previous study reported that ECL efficiently sensitized X-ray-induced apoptosis of NSCLC A549 and COR-L23 cells by inducing cell cycle arrest at the G2/M phase and inhibiting the repair of radiation-induced DNA double-strand breaks (21). Notably, ECL was also reported to inhibit the NF- κ B activity in TNF- α -activated 293/NF- κ B-luc cells, a stable cell line containing an NF- κ B driven luciferase reporter gene (22). Another study suggested that ECL may act as a protein synthesis inhibitor, which suppressed the expression of the NF- κ B-dependent target genes ICAM-1, VCAM-1 and E-selectin in TNF α -activated human endothelial cells (23). It was therefore hypothesized that ECL may exert an anticancer effect by suppressing the AKT/NF- κ B signaling pathway in human NSCLC cells, leading to the induction of apoptosis and the enhancement of cisplatin-induced cytotoxicity.

Materials and methods

Chemicals, reagents and antibodies. ECL, with a purity of 93.6%, was purchased from Biopurify Phytochemicals Ltd. Cisplatin was purchased from Merck KGaA. Culture media Roswell Park Memorial Institute (RPMI)-1640, Dulbecco's modified Eagle's medium (DMEM), penicillin/streptomycin antibiotics and trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA) were purchased from Thermo Fisher Scientific, Inc. Fetal bovine serum (FBS) was obtained from GE

Healthcare Life Sciences. MTT was obtained from AppliChem GmbH. Muse[®] Cell Cycle kit and Muse[™] Annexin V & Dead Cell kit were purchased from EMD Millipore. Mammalian Protein Extraction buffer was purchased from GE Healthcare Life Sciences. Antibodies specific to AKT (product no. 9272) and phosphorylated (p)-AKT (S473; product no. 9271), NF- κ B p65 (product no. 6956), p-NF- κ B-p65 (S536; product no. 3033), caspase-3 (product no. 9662), cleaved caspase-3 (Asp175; product no. 9661), poly (ADP-ribose) polymerase (PARP; product no. 9542) and survivin (product no. 2808) were purchased from Cell Signaling Technology, Inc. Antibodies against Bcl-xL (product code ab32370 and β -actin (cat. no. A2066) were obtained from Abcam and Merck KGaA, respectively. Horseradish peroxidase-conjugated goat anti-rabbit (cat. no. 1706515) or goat anti-mouse (cat. no. 1706516) immunoglobulin G were purchased from Bio-Rad Laboratories, Inc. Cisplatin was dissolved in normal saline (1 mg/ml) and maintained at room temperature. ECL was dissolved in dimethyl sulfoxide (DMSO) and stored at -20°C until use. The final concentration of DMSO was <0.5% (v/v), which was also present in the corresponding controls at the same concentrations.

Cell lines and cell culture. The human adenocarcinoma NSCLC A549 cell line was obtained from the American Type Culture Collection. The human lung squamous cell carcinoma Calu-1 cell line was purchased from the CLS Cell Lines Service. The A549 and Calu-1 cells were grown and maintained in DMEM and RPMI-1640 media, respectively. These culture media were supplemented with 10% (v/v) FBS, 100 U/ml penicillin and 100 μ g/ml streptomycin. All cell lines were incubated at 37°C in a humidified atmosphere containing 5% CO₂. All cell lines were mycoplasma-free. The continuous cell lines were routinely checked every other month by PCR using a service from the Center for Veterinary Diagnosis, Faculty of Veterinary Science, Mahidol University Salaya Campus, Nakorn Pathom (Thailand).

Cell viability assay. The A549 (3,500 cells/well) and Calu-1 (6,000 cells/well) cells were seeded on a 96-well plate for 24 h and then exposed to 0, 2.5, 5, 10, 20, 40, 80 and 160 μ M cisplatin or ECL for 24 and 48 h. The number of viable cells was determined by MTT assay, as previously described (24). The optical density (OD) of the formazan dye was measured at 570 nm using a microplate reader (Bio-Rad Laboratories, Inc.). The percentage of cell viability was calculated using the formula: Cell viability = (OD_{treated}/OD_{control}) \times 100%. The ECL concentrations required for 20, 40 and 50% inhibition of cell viability (IC₂₀, IC₄₀ and IC₅₀, respectively) in A549 and Calu-1 cells at 24 h were then determined from the viability curves and selected for further experiments.

Colony formation assay. The antiproliferative effects of ECL on human NSCLC A549 and Calu-1 cell lines were confirmed using a colony formation assay. Briefly, the A549 (2 \times 10⁵ cells/well) or Calu-1 (3 \times 10⁵ cells/well) cells, were seeded on a 6-well plate and incubated for 24 h. The cells were then treated with ECL at an IC₂₀, IC₄₀ and IC₅₀ concentration for 24. Next, the cells were collected by trypsinization with 0.25% trypsin-EDTA (Gibco; Thermo Fisher Scientific,

Inc.) and replated in 10-cm culture dishes at a low density of 400 cells/dish to allow colony formation for 2 weeks, with a change of the fresh growth media every 3 days. The colonies were washed with cold phosphate-buffered saline (PBS), fixed with 100% methanol for 10 min, and stained with 0.5% crystal violet for 1 h at room temperature. The colonies containing >50 cells were counted using an inverted microscope at a magnification of x40.

Cell cycle analysis by flow cytometry. The A549 (2×10^5 cells/well) or Calu-1 (3×10^5 cells/well) cells, were seeded on a 6-well plate and incubated for 24 h. Following ECL treatment at the indicated concentrations and incubation times, cells were collected, fixed gently in 70% ethanol and stored at -20°C overnight. The fixed cells were then stained using a Muse[®] Cell Cycle kit for 30 min at room temperature in the dark. The cell cycle phase distributions were determined by Muse[®] Cell Analyzer and Muse[®] Cell Cycle software module (version 1.0.0.0.; EMD Millipore). The cell cycle phases subG1, G1, S and G2/M were analyzed using GuavaSoft 2.7 software (EMD Millipore).

Combination index (CI) analysis for combined treatment with ECL and cisplatin. The IC_{20} and IC_{40} of ECL were selected to further study cisplatin co-treatment, to compare the effects of sub-cytotoxic and highly toxic concentrations of ECL on cisplatin sensitization. A549 or Calu-1 cells were treated with 0-160 μM cisplatin either alone or in combination with ECL at the IC_{20} (2 or 10 μM , respectively) or the IC_{40} (12 or 80 μM , respectively) for 24 h. Cell viability was then determined by MTT assay, as previously described. The CI, which reflects the nature of drug interactions in combination chemotherapy, was calculated using the Chou-Talalay Method (25) based on the following equation: $\text{CI} = [(D)_1 / (Dx)_1] + [(D)_2 / (Dx)_2]$, where $(D)_1$ and $(D)_2$ represent the concentrations of compounds 1 (Cisplatin) or 2 (ECL) in the co-treatment that attains a 50% inhibition, and $(Dx)_1$ and $(Dx)_2$ represent the concentrations of compounds 1 or 2 in the co-treatment that attains a 50% inhibition when present alone (26). A CI of <0.9, 0.9-1.1 and >1.1 indicated synergistic, additive and antagonistic effects, respectively.

Cell apoptosis assay by flow cytometry. The A549 (2×10^5 cells/well) or Calu-1 (3×10^5 cells/well) cells were seeded on a 6-well plate and incubated for 24 h. The cells were then treated with ECL alone, cisplatin alone or ECL (IC_{20} or IC_{40}) plus cisplatin for 24 h. Apoptosis was quantified by staining with Muse[®] Annexin V & Dead Cell kit (EMD Millipore) for 20 min at room temperature in the dark, according to the manufacturer's instructions. The quantitative analysis of cell living, early and late apoptosis, and cell death, were obtained by the Muse[®] Cell Analyzer (EMD Millipore) using the Muse[®] Annexin V & Dead Cell software module (version 1.0.0.0) with a minimum of 2,000 events per sample.

Western blotting. Protein expression levels of p^(S473)-AKT, AKT, p^(S536)-NF- κ B p65, NF- κ B p65, caspase-3, PARP, Bcl-xL and survivin were measured by immunoblotting, as described in our previous study (27). Briefly, cells collected from each treatment group were lysed in a Mammalian Protein

Extraction buffer. Protein concentration was determined using the Bradford assay. Protein (30 μg) from each sample was separated by 12% SDS-PAGE and electro-transferred to PVDF membranes. Primary antibodies (1:1,000 dilution) were added and incubated at 4°C overnight; after which the appropriate HRP-conjugated secondary antibody (1:5,000 dilution) was added and incubated for 2 h at room temperature. Chemiluminescence signals were detected on the X-ray film. As an internal control, the β -actin primary antibody was also probed. The normalized mean density of the immunological cross-reactive band was quantified by ImageJ software (version 1.51j8; National Institutes of Health).

Statistical analysis. Data are expressed as the mean \pm standard deviation of at least three independent experiments. Significant differences among groups were analyzed and compared by one-way analysis of variance and Tukey's post hoc test using SPSS 22.0 software (IBM Corp.). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

ECL inhibits the cell viability and colony-forming capacity of NSCLC cells. To determine the effect of ECL on NSCLC cell viability *in vitro*, human lung cancer A549 (adenocarcinoma) and Calu-1 (squamous cell carcinoma) cells were incubated with various concentrations of ECL (0-160 μM) for 24 or 48 h, and cell viability was then examined by MTT assay. As revealed in Fig. 1B and C, ECL treatment significantly reduced the cell viability of both NSCLC cell lines in a concentration- and time-dependent manner. The ECL concentrations required for a 50% inhibition of cell viability (IC_{50}) of A549 cells at 24 and 48 h (20.81 ± 1.86 and 3.15 ± 0.36 μM , respectively) were markedly lower compared with those required for the same inhibition of cell viability in Calu-1 cells (151.87 ± 4.75 and 12.95 ± 0.85 μM , respectively), indicating that ECL has a higher toxicity in A549 than Calu-1 cells. Table I summarizes the ECL concentrations at IC_{20} , IC_{40} and IC_{50} after 24 and 48 h of incubation. The IC_{20} , IC_{40} and IC_{50} concentrations of ECL at 24 h (2, 12 and 20 μM for A549 cells, and 10, 80 and 150 μM for Calu-1 cells) were used for subsequent experiments.

Subsequently, the anti-proliferative effect of ECL on the A549 and Calu-1 cells was confirmed by the inhibition of colony formation, as revealed in Fig. 1D and E. The indicated cells were treated with ECL at the IC_{20} , IC_{40} , and IC_{50} concentrations for 24 h before the cells were replated (300 cells/plate) and allowed to form colonies. The numbers of the colonies formed proportionally reflect the IC_{20} , IC_{40} and IC_{50} concentrations of ECL.

ECL induces cell cycle arrest in NSCLC cells. To investigate the mechanism through which ECL inhibits cell growth, cell cycle distribution was analyzed by flow cytometry following 24 h of ECL treatment with the IC_{20} , IC_{40} and IC_{50} concentrations. As revealed in Fig. 2A and C, ECL caused cell cycle arrest at the G2/M phase in A549 cells. In Calu-1 cells, ECL caused the S phase arrest when treated with IC_{20} , IC_{40} or IC_{50} concentrations (Fig. 2B and D) and induced both S and G2/M phase arrest when treated with the IC_{20} concentration. Such arrest in both NSCLC cells was associated with a concomitant

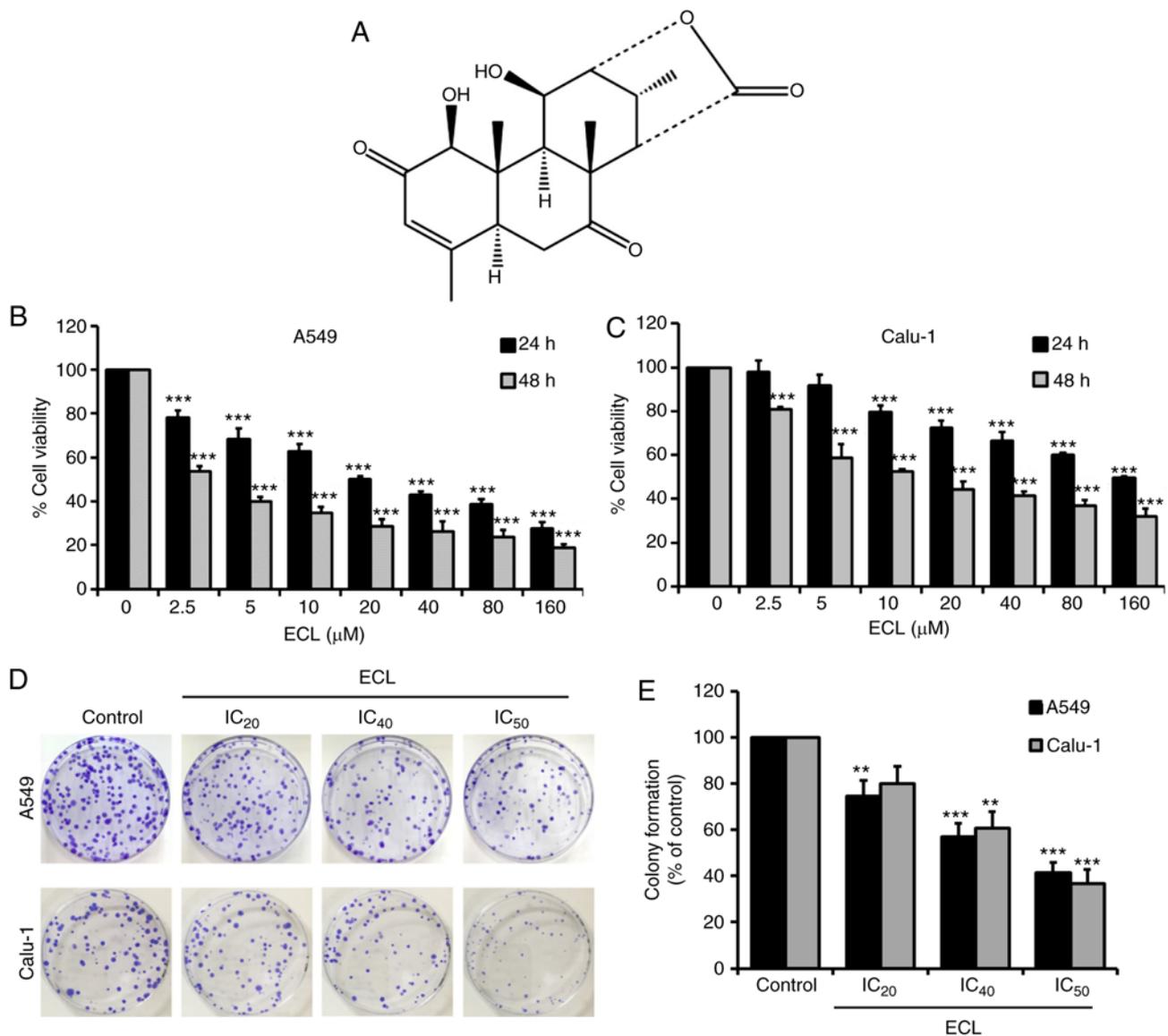


Figure 1. Anticancer effects of ECL on human NSCLC cell viability and colony formation. (A) Chemical structure of ECL. The viability of (B) A549 and (C) Calu-1 cells treated with indicated concentrations of ECL for 24 and 48 h was examined by MTT assay. Data were expressed as a percentage of viable cells relative to the control. (D) Representative images of the A549 and Calu-1 cell colonies following treatment with ECL at the IC₂₀, IC₄₀ and IC₅₀ concentrations. (E) Bar graphs represent the percentage of colony formation of the A549 and Calu-1 cells normalized to the control groups. All data are presented as the mean \pm SD from three independent experiments. ** $P < 0.01$, *** $P < 0.001$ vs. the non-treated control. ECL, eurycomalactone; NSCLC, non-small cell lung cancer.

decrease in the percentage of cells at the G1 phase. Moreover, the accumulation of a sub-G1 population, which comprised apoptotic cells containing only fractional DNA content, was observed in a dose-dependent manner in the A549 and Calu-1 cells. These results indicated that ECL could induce NSCLC cell death following the induction of cell cycle arrest.

ECL promotes NSCLC cell apoptosis. The effect of ECL on the NSCLC cell apoptosis was confirmed using Annexin V-FITC/7-AAD double-staining, followed by flow cytometry (Fig. 3A and B). Following the 24-h ECL treatment, the apoptotic rates of A549 (Fig. 3C) and Calu-1 (Fig. 3D) cells in the ECL-treated group were significantly increased in a dose-dependent manner, when compared with the control group. Next, the expression of apoptosis regulators, including the pro-apoptotic caspase-3 and PARP proteins,

and the anti-apoptotic Bcl-xL and survivin proteins, was determined by immunoblotting in the A549 and Calu-1 cells (Fig. 3E and F, respectively). The expression levels of active caspase-3 and active PARP (cleaved form) were both markedly induced, while that of Bcl-xL and survivin were significantly decreased following treatment with various concentrations of ECL for 24 h, when compared with the control group. These results confirmed that ECL could trigger apoptotic cell death in NSCLC cells.

ECL suppresses AKT/NF- κ B activation in NSCLC cells. The inhibitory effect of ECL on the viability of NSCLC cells by triggering apoptotic cell death prompted us to investigate the AKT/NF- κ B signaling pathway, which participates in not only multiple steps of lung cancer progression, but also the resistance to chemotherapy (28,29). Phosphorylation at serine 473

Table I. Cytotoxic effect of ECL against two different types of NSCLC cells (A549 and Calu-1).

NSCLC cell lines	Subtypes	24 h			48 h		
		ECL (μ M)			ECL (μ M)		
		IC ₂₀	IC ₄₀	IC ₅₀	IC ₂₀	IC ₄₀	IC ₅₀
A549	Adenocarcinoma	2.29±0.44	12.02±1.44	20.81±1.86	1.04±0.19	2.12±0.16	3.15±0.36
Calu-1	Squamous cell carcinoma	10.14±1.67	80.77±6.84	156.30±5.95	2.60±0.12	5.09±0.65	12.95±0.85

IC₂₀, IC₄₀ and IC₅₀, the concentrations required for 20, 40 and 50% inhibition of cell viability. The values (μ M) are expressed as the mean \pm SD of three independent experiments. ECL, eurycomalactone; NSCLC, non-small cell lung cancer.

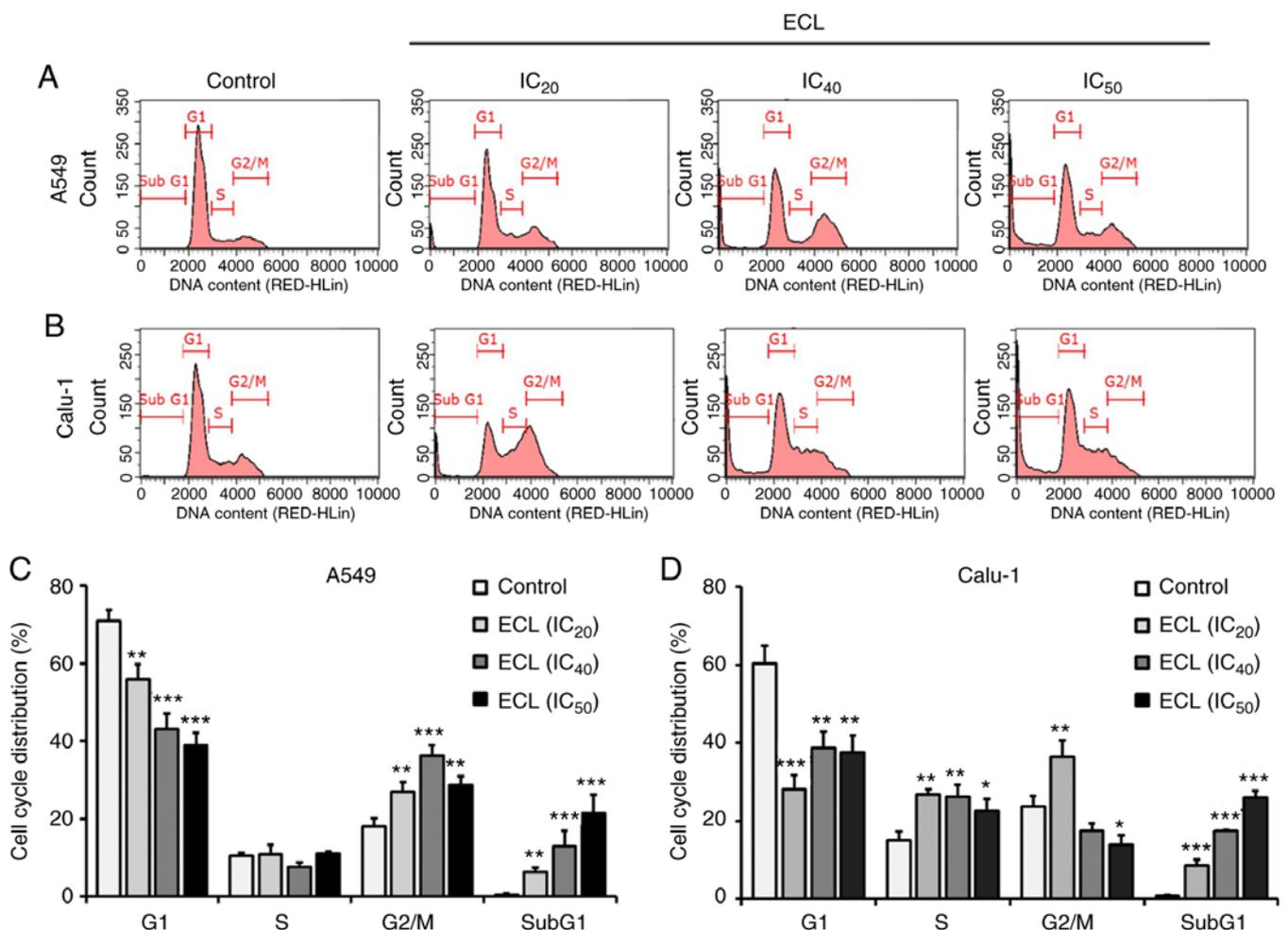


Figure 2. Cell cycle arrest induction in human NSCLC cells treated with ECL. The representative histograms of the cell cycle distribution in (A) A549 and (B) Calu-1 cells. The quantitative analysis of the cell cycle phase distribution in (C) A549 and (D) Calu-1 cells treated with ECL at the IC₂₀, IC₄₀ and IC₅₀ concentrations for 24 h. The cell cycle was analyzed by PI staining-flow cytometry. Data are indicated as the mean \pm SD. *P<0.05, **P<0.01, ***P<0.001 vs. the untreated control group. NSCLC, non-small cell lung cancer; ECL, eurycomalactone.

(S473) in the C-terminal hydrophobic motif of AKT is involved in AKT activation (30), and phosphorylation at the serine 536 (S536) position of the NF- κ B p65 subunit is required for the activation and nuclear translocation of NF- κ B (31). ECL significantly inhibited the expression levels of p^(S473)-AKT, total AKT,

p^(S536)-NF- κ B p65 and total NF- κ B p65 in both A549 (Fig. 4A) and Calu-1 (Fig. 4B) cells. In fact, ECL downregulated the expression of p^(S473)-AKT and total AKT in both A549 and Calu-1 cells when normalized to β -actin (Fig. S1). Likewise, the significant depletion of both p^(S536)-NF- κ B p65 and total

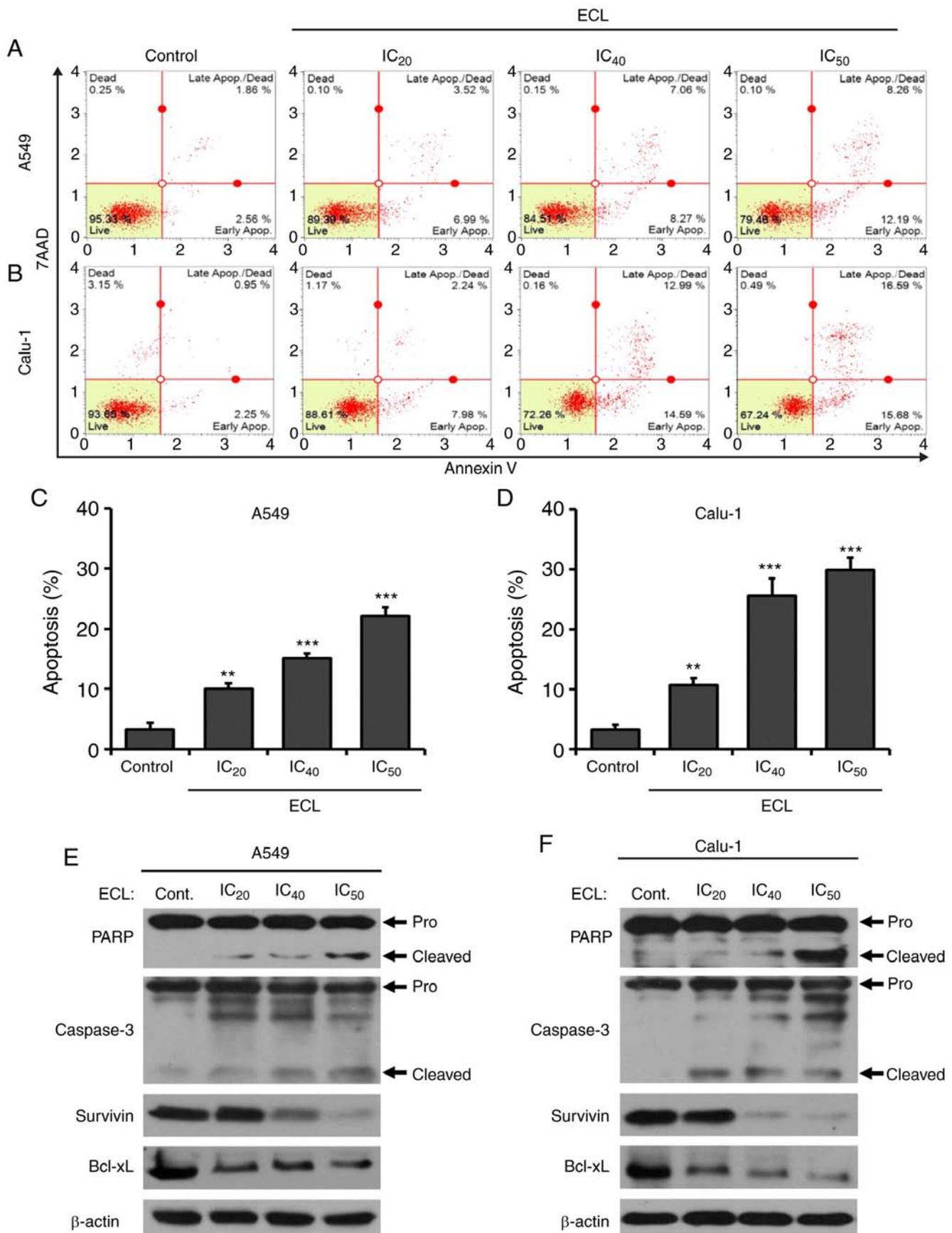


Figure 3. Apoptosis induction in human NSCLC cells treated with ECL. The representative dot plots of (A) A549 and (B) Calu-1 cell apoptosis which were analyzed by Annexin V-FITC/7AAD double-staining followed by flow cytometry. The quantitative analysis of total apoptotic cell percentage in (C) A549 and (D) Calu-1 cells treated with ECL at the IC₂₀, IC₄₀ and IC₅₀ concentrations for 24 h. Data is indicated as the mean ± SD. **P<0.01, ***P<0.001 vs. the untreated control group. The expression of apoptotic related-proteins including PARP, caspase-3, survivin, and Bcl-xL was detected in (E) A549 and (F) Calu-1 cells by western blot analysis following treatment with ECL at the IC₂₀, IC₄₀ and IC₅₀ concentrations for 24 h. β-actin was used as an internal control. NSCLC, non-small cell lung cancer; ECL, eurycomalactone; PARP, poly (ADP-ribose) polymerase.

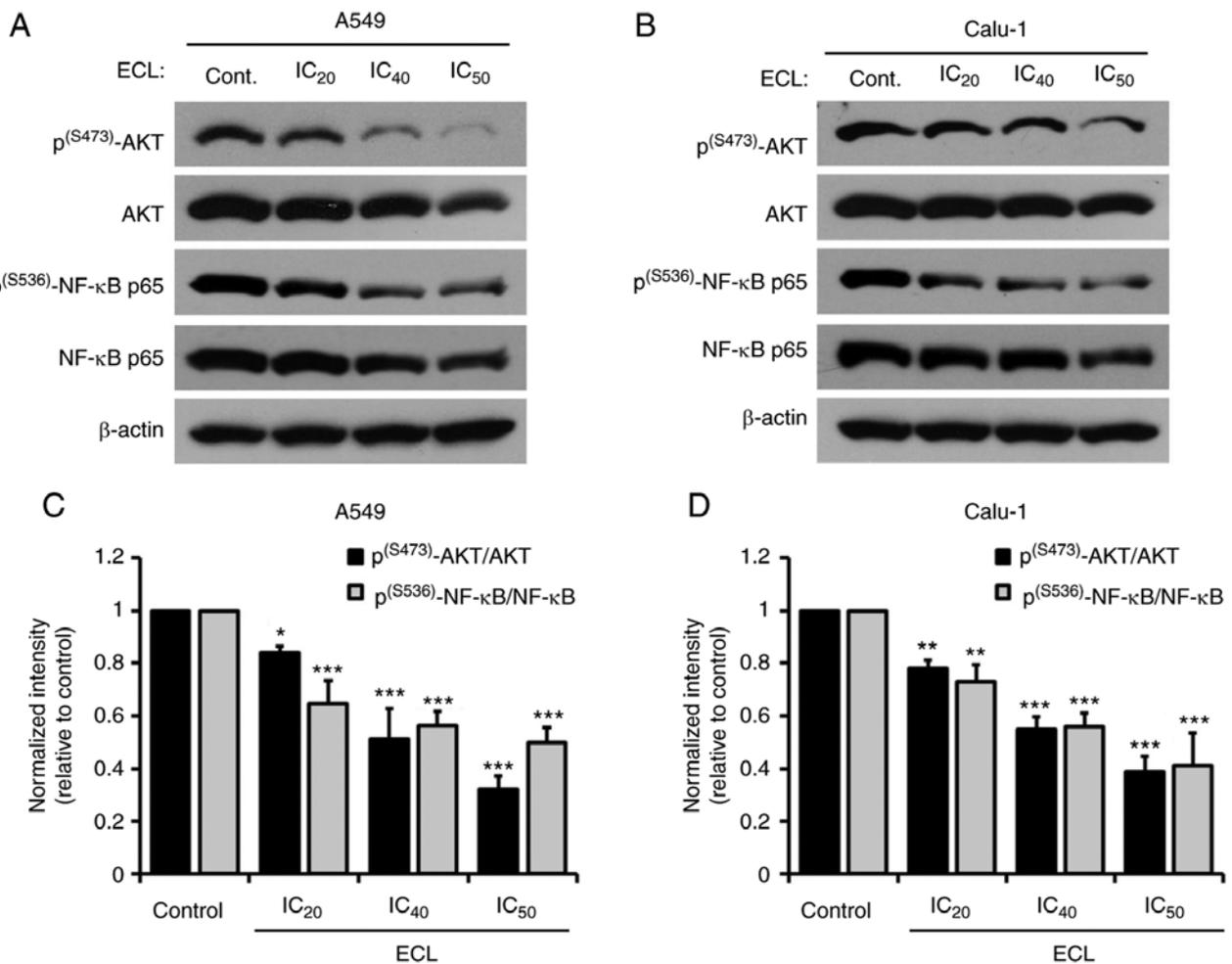


Figure 4. Inhibitory effect of ECL on AKT/NF-κB phosphorylation in human NSCLC cells. The representative immunoblots of p^(S473)-AKT, AKT, p^(S536)-NF-κB p65 and NF-κB p65 in whole cell extracts of (A) A549 and (B) Calu-1 cells treated with ECL at the IC₂₀, IC₄₀ and IC₅₀ concentrations for 24 h. β-actin was used as an internal control. The ratio of p^(S473)-AKT/AKT and that of p^(S536)-NF-κB p65/NF-κB p65 expression were normalized to control groups in (C) A549 and (D) Calu-1 cells. Data are presented as the mean values ± SD. *P<0.05, **P<0.01, ***P<0.001 vs. the untreated control group. ECL, eurycomalactone; AKT, protein kinase B; NF-κB, nuclear factor-κB; NSCLC, non-small cell lung cancer.

NF-κB p65 levels when normalized to β-actin was observed in the NSCLC cells treated with ECL (Fig. S2). Notably, the ratio of p^(S473)-AKT/AKT and p^(S536)-NF-κB p65/NF-κB p65 were also significantly decreased in the A549 (Fig. 4C) and Calu-1 (Fig. 4D) cells, supporting the involvement of AKT/NF-κB inactivation in ECL-induced apoptosis in the NSCLC cells.

Cisplatin treatment induces the phosphorylation of AKT and NF-κB at the activation sites. To observe their cisplatin sensitivity, the A549 and Calu-1 cells were treated with cisplatin at various concentrations for 24 h, and cell viability was determined by MTT assay. As revealed in Fig. 5A, the A549 cells displayed a 2-fold greater sensitivity to cisplatin than the Calu-1 cells, when comparing their IC₅₀ values. Next, it was determined whether cisplatin treatment (at IC₂₀) could stimulate AKT/NF-κB signaling in the NSCLC cells using immunoblotting of p^(S473)-AKT, total AKT, p^(S536)-NF-κB p65 and total NF-κB p65. The results in Fig. 5B revealed that the levels of p^(S473)-AKT and p^(S536)-NF-κB p65 were markedly increased in the A549 and Calu-1 cells following cisplatin treatment. The highest levels of p^(S473)-AKT and p^(S536)-NF-κB p65 were detected at 30 min and 24 h, respectively, following

cisplatin treatment in both NSCLC cell lines. Therefore, the activation of AKT and NF-κB was responsive to cisplatin treatment in the NSCLC cells.

ECL significantly enhances cisplatin sensitivity in NSCLC cells. Next, it was examined whether the combination of ECL and cisplatin exerts a lethal enhancement in NSCLC cells. Following co-treatment with the IC₂₀ or IC₄₀ concentrations of ECL and various concentrations of cisplatin for 24 h, MTT assays were performed. As demonstrated in Fig. 6A and B, the co-treatment significantly reduced the viability of both NSCLC cells when compared with cisplatin alone. Table II summarizes the IC₅₀ values of cisplatin in the co-treatment with ECL (IC₂₀ or IC₄₀); the CI values were calculated to define the drug interaction of cisplatin and ECL, when administered in combination, as synergistic, additive or antagonistic effects. Co-treatment with ECL led to the positive dose (IC₅₀) reduction of cisplatin in both NSCLC cell lines. The CI values of cisplatin combined with ECL at the IC₂₀ and IC₄₀ concentrations in the A549 cells were 0.66±0.06, indicating synergistic effects, and 0.91±0.04, indicating additive effects (Fig. 6C). Moreover, the CI values of cisplatin plus ECL at the IC₂₀ and

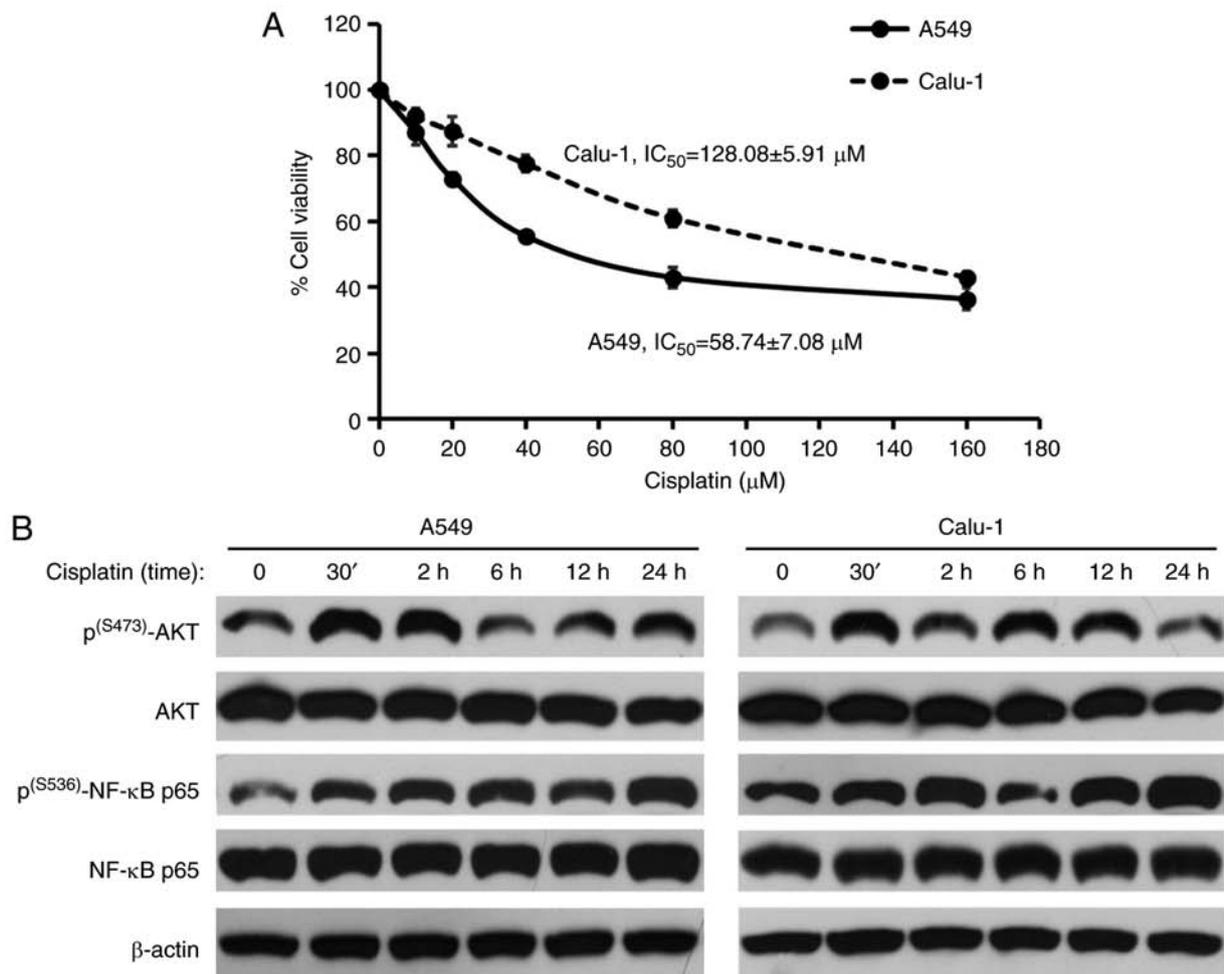


Figure 5. Cisplatin-induced enhancement of AKT/NF- κ B signaling activation in human NSCLC cells. (A) The effect of cisplatin on cell viability of the A549 and Calu-1 cells at 24 h of treatment assessed by MTT assay. Data are presented as the mean values \pm SD. (B) The representative immunoblots of p^(S473)-AKT, AKT, p^(S536)-NF- κ B p65 and NF- κ B p65 in whole cell extracts of the A549 and Calu-1 cells treated with cisplatin at the IC₂₀ concentration for indicated time-points. β -actin was used as an internal control. AKT, protein kinase B; NF- κ B, nuclear factor- κ B; NSCLC, non-small cell lung cancer.

IC₄₀ concentrations in the Calu-1 cells were 0.60 ± 0.04 and 0.73 ± 0.03 , respectively, indicating their synergistic effects (Fig. 6D).

Combination of cisplatin with ECL enhances apoptosis induction in NSCLC cells. Since the effect of ECL on potentiating cisplatin sensitivity was found in the A549 and Calu-1 cells, the mechanisms of ECL on the enhancement of cisplatin cytotoxicity were further investigated by examining the induction of apoptosis. The flow cytometric results presented in Fig. 7A and B revealed that co-treatment of cisplatin at the IC₂₀ value with ECL at the IC₂₀ or IC₄₀ concentrations significantly induced a higher number of A549 cells to undergo apoptosis compared with either cisplatin or ECL alone. Similar results were obtained in Calu-1 cells (Fig. 7C and D). The effect on apoptosis was confirmed by immunoblotting of pro-apoptotic and anti-apoptotic proteins. As revealed in Fig. 7E and F, caspase-3 cleavage was induced by either ECL or cisplatin as a single agent, whereas the co-treatment resulted in a higher increase of cleaved caspase-3, with a corresponding decrease in the pro-form of caspase-3. By contrast, the co-treatment of cisplatin with ECL markedly decreased the expression of anti-apoptotic Bcl-xL and survivin proteins, when compared

with cisplatin alone. These findings demonstrated that the combination of cisplatin and ECL treatment further induced apoptosis in the NSCLC cells more effectively than cisplatin alone.

ECL inhibits cisplatin-induced AKT and NF- κ B phosphorylation in NSCLC cells. To understand the cisplatin sensitization mechanisms of ECL, our attention turned to the cisplatin resistance-related AKT/NF- κ B signaling pathway, as ECL alone could suppress AKT/NF- κ B activation in the NSCLC cells, as demonstrated in the previous results (Fig. 4). The expression of p^(S473)-AKT, total AKT, p^(S536)-NF- κ B p65 and total NF- κ B p65 was therefore detected by western blotting in the A549 (Fig. 8A) and Calu-1 (Fig. 8B) cells following treatment with ECL or cisplatin alone, or in combination. ECL alone could significantly suppress the ratio of p^(S473)-AKT/AKT (Fig. 8C and D) and p^(S536)-NF- κ B p65/NF- κ B p65 (Fig. 8E and F) in the A549 and Calu-1 cells, when compared to the non-treatment control. On the other hand, cisplatin alone significantly increased the ratio of p^(S473)-AKT/AKT and p^(S536)-NF- κ B p65/NF- κ B p65 in both tested NSCLC cells, indicating that cisplatin could induce AKT and NF- κ B activation. Notably, the co-treatment of ECL with cisplatin significantly decreased

Table II. IC₅₀ values of cisplatin in NSCLC cells when administered alone or in combination with ECL, the CI and drug interactions of ECL and cisplatin combination.

NSCLC cell lines	Treatment	IC ₅₀ (μM)	Combination index (CI)	Drug interactions
A549	Cisplatin	53.58±3.05	-	-
	Cisplatin + ECL(IC ₂₀)	30.16±2.62	0.66±0.06	Synergism
	Cisplatin + ECL(IC ₄₀)	17.91±2.11	0.91±0.04	Additive
Calu-1	Cisplatin	135.49±7.83	-	-
	Cisplatin + ECL(IC ₂₀)	72.49±2.05	0.60±0.04	Synergism
	Cisplatin + ECL(IC ₄₀)	29.53±3.71	0.73±0.03	Synergism

IC₅₀, the concentrations required for 50% inhibition of cell viability. The values (μM) are expressed as the mean ± SD of three independent experiments. CI were analyzed using the Chou-Talalay method which reflects drug interactions; CI <0.9, CI=0.9-1.1, and CI >1.1 indicated synergistic, additive, and antagonistic effects, respectively. NSCLC, non-small cell lung cancer; ECL, eurycomalactone; CI, combination index.

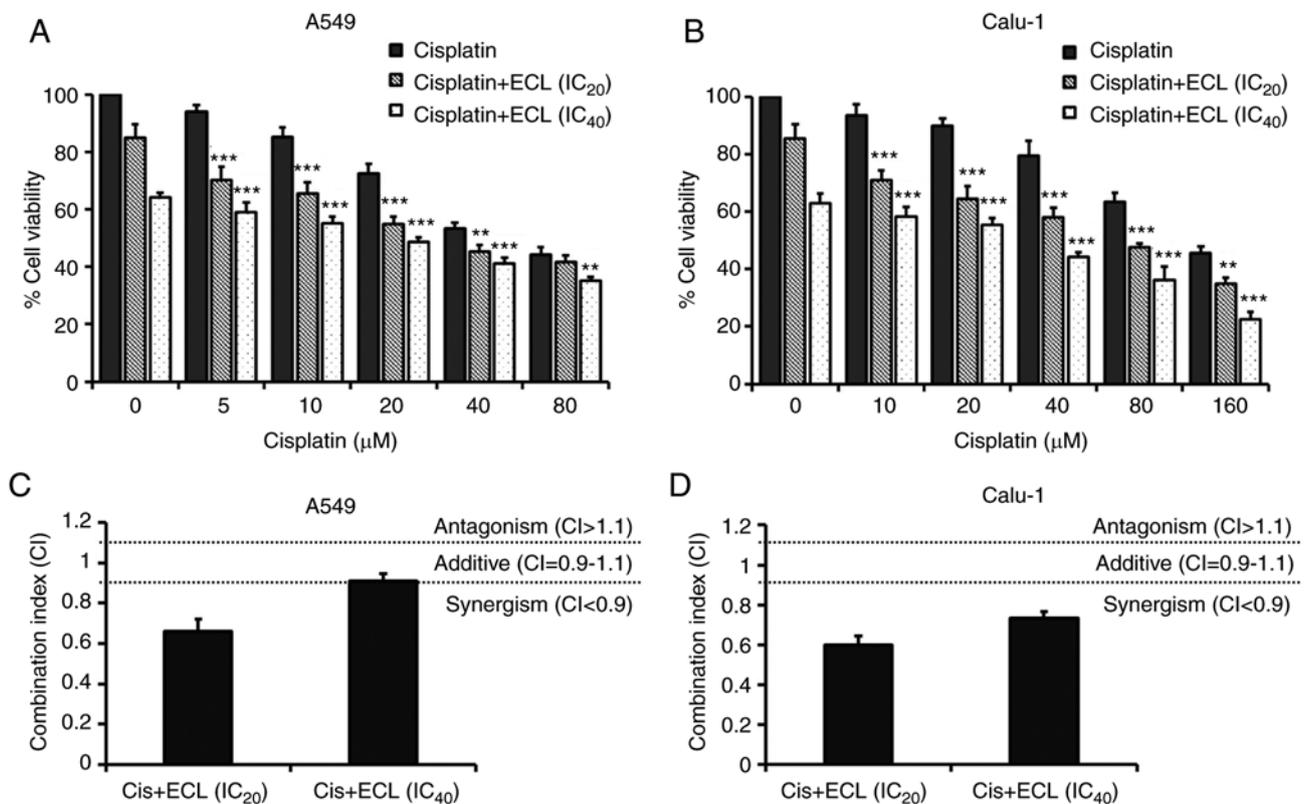


Figure 6. Cisplatin-sensitizing effects of ECL on human NSCLC cells. (A) The A549 and (B) Calu-1 cells were treated for 24 h with 0-160 μM of cisplatin either alone or in combination with ECL at the IC₂₀ or IC₄₀ concentrations. The cell viability was determined by MTT assay. The mean CI values of cisplatin-ECL combination in (C) A549 and (D) Calu-1 cells. CI <0.9 designates synergism, CI=0.9-1.1 indicates additivity, and CI >1.1 represents antagonism (denoted by the dashed line). All data are presented as the mean ± SD. **P<0.01, ***P<0.001 compared with each concentration of cisplatin alone. ECL, eurycomalactone; NSCLC, non-small cell lung cancer; CI, combination index.

the ratio of p^(S473)-AKT/AKT and p^(S536)-NF-κB p65/NF-κB p65, when compared to cisplatin alone. In combination, ECL sensitized the cisplatin-induced cytotoxicity in NSCLC cells at least partially by inhibiting the activation of the AKT/NF-κB signaling pathway.

Discussion

As a leading cause of cancer-related mortality worldwide, lung cancer has the greatest annual burden among all types

of cancer (32). The development of novel agents for the treatment of lung cancer is urgently required. Plant-derived compounds with diverse bioactivities have attracted increasing attention for their pharmaceutical potential in cancer treatment, by being used either alone or as part of combination therapy, to potentiate the effect of chemotherapeutic drugs (12). The potential for plant extracts to act as anticancer therapeutic agents is due to their abilities to promote apoptosis and inhibit tumor growth and metastasis with few side effects (33).

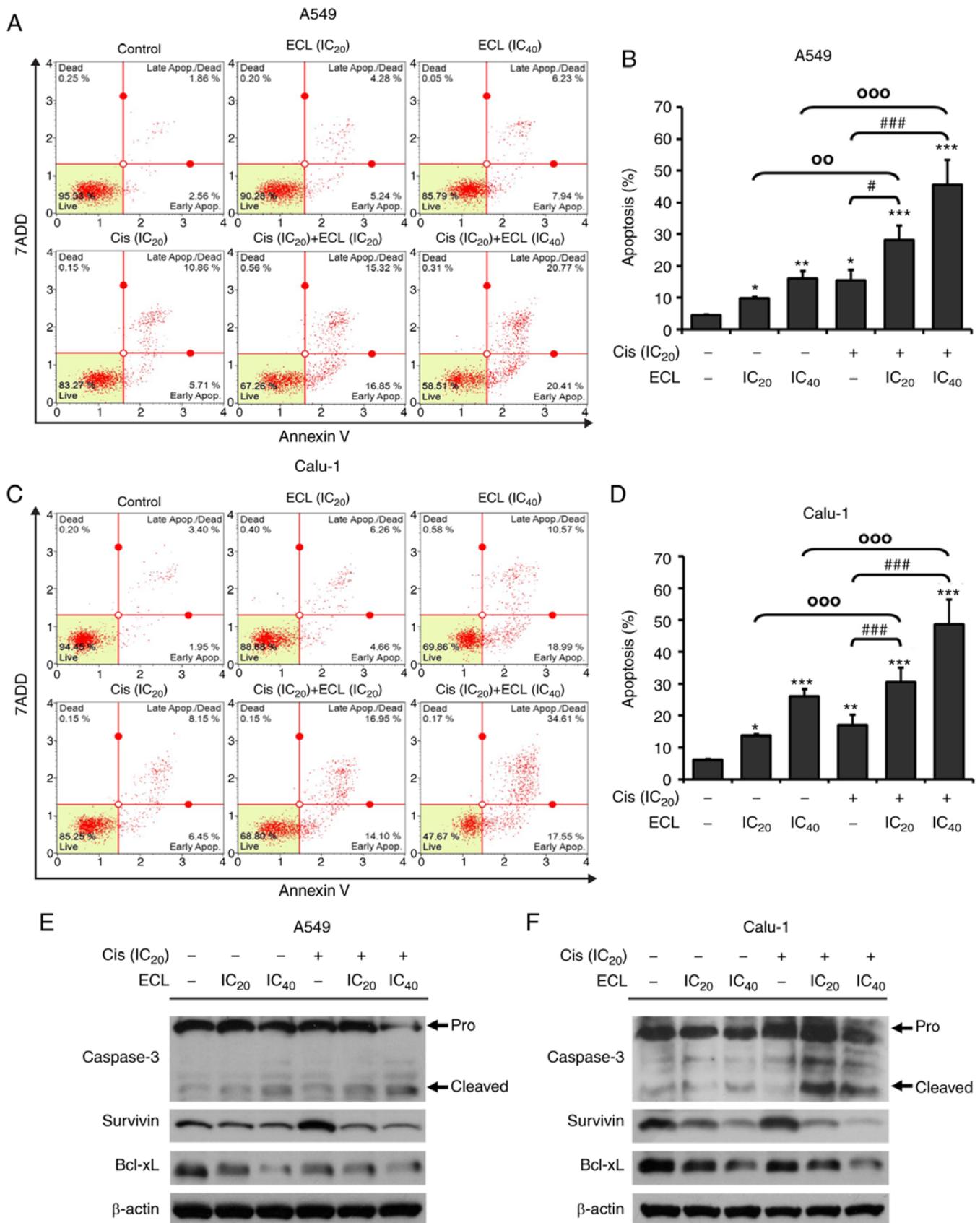


Figure 7. Effect of cisplatin and ECL alone or in the combination on apoptosis induction of NSCLC cells. (A) The representative dot plots display the apoptosis and (B) the quantitative analysis of total apoptotic cell percentage in the A549 cells treated with ECL at IC₂₀ or IC₄₀ alone, cisplatin at IC₂₀ alone, or in the combination for 24 h. (C) The representative dot plots and (D) the quantitative analysis of total apoptotic cells percentage in the Calu-1 cells treated with the indicated agents. Cell apoptosis was analyzed by Annexin V-FITC/7AAD double-staining with flow cytometry. *P<0.05, **P<0.01, ***P<0.001 vs. the control (non-treatment group); #P<0.05, ###P<0.001 vs. cisplatin alone; °P<0.01, °°P<0.001 vs. ECL alone at each concentration. Expression of caspase-3, survivin, and Bcl-xL proteins was detected in (E) A549 and (F) Calu-1 cells by western blot analysis following 24 h of treatment with cisplatin alone or the IC₂₀ or IC₄₀ concentrations of ECL alone, or both agents. β-actin was used as an internal control. ECL, eurycomalactone; NSCLC, non-small cell lung cancer.

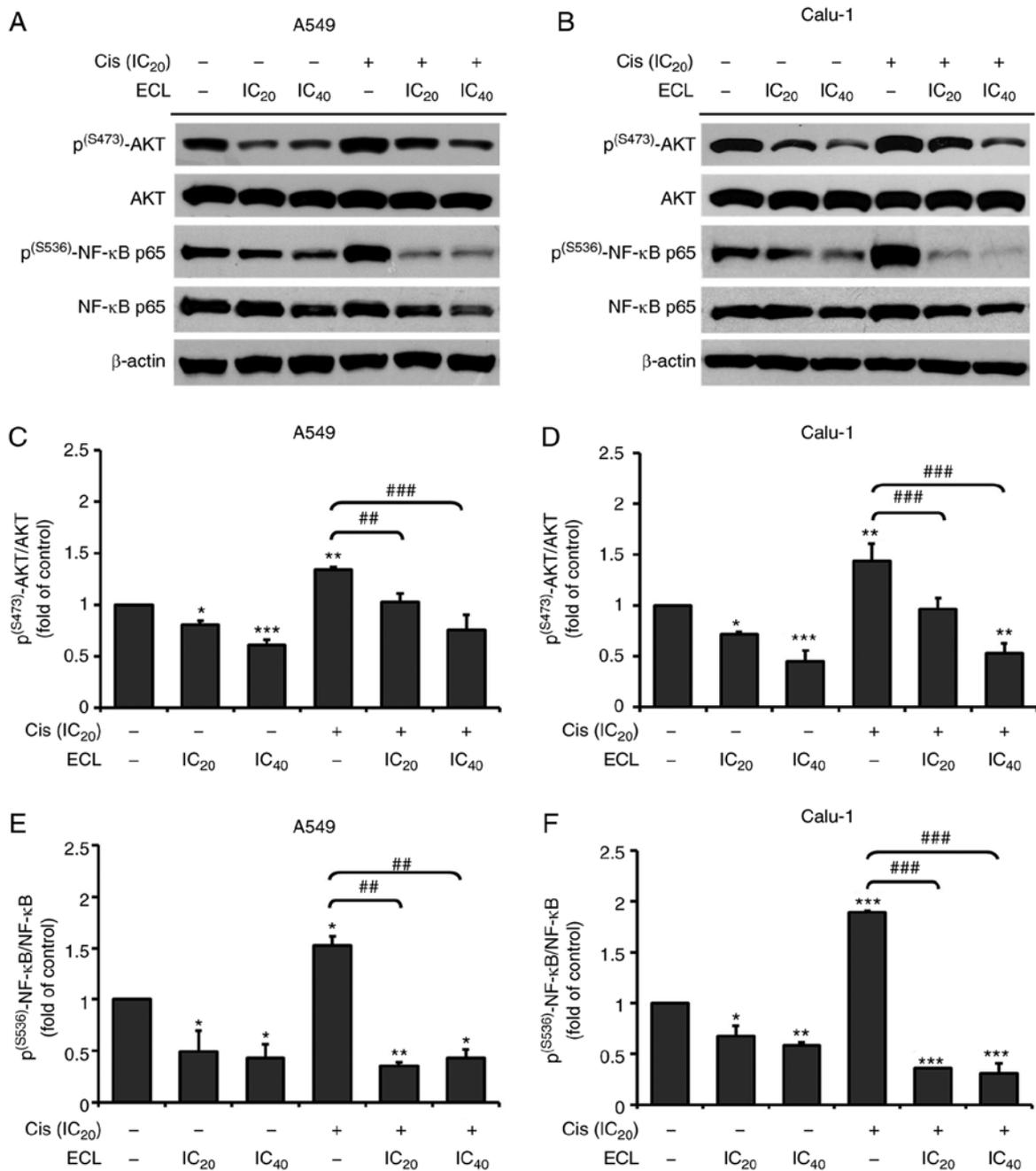


Figure 8. Inhibitory effect of ECL on cisplatin-induced AKT/NF-κB signaling activation in human NSCLC cells. The A549 and Calu-1 cells were treated with cisplatin at the IC₂₀ or ECL at the IC₂₀ or IC₄₀ concentrations alone, or in combination for 24 h. Representative immunoblotting images of (A) A549 and (B) Calu-1 cells stained for p^(S473)-AKT, AKT, p^(S536)-NF-κB p65 and NF-κB p65. β-actin was included as an internal control. Bar graphs revealed the average relative expression of (C and D) p^(S473)-AKT/AKT ratio and (E and F) p^(S536)-NF-κB p65/NF-κB p65 ratio in the A549 and Calu-1 cells, respectively. *P<0.05, **P<0.01, ***P<0.001 vs. the control (non-treatment group); ##P<0.01; ###P<0.001 vs. cisplatin alone. ECL, eurycomalactone; AKT, protein kinase B; NF-κB, nuclear factor-κB; NSCLC, non-small cell lung cancer.

In the screening of cytotoxicity, ECL, a natural active quassinoid from *E. longifolia* Jack, demonstrated a strong cytotoxicity activity toward various human cancer cell types including human breast cancer MCF-7 and human NSCLC A549 cell lines (19,20). Herein, we attempted to elucidate the anticancer effect of ECL on the survival, proliferation, apoptosis and cisplatin sensitization in NSCLC A549 and Calu-1 cells, as well as the related cell signaling mechanism. Another quassinoid compound of the *E. longifolia* Jack family, eurycomanone, has been reported to have an anticancer mechanism,

through which it decreased the activity of prohibitin in lung cancer cells (34) and the expression of p53 in hepatocellular carcinoma cells (35). Both proteins regulate the cell cycle, proliferation and apoptosis. Moreover, eurycomanone was revealed to act on leukemia cells by inhibiting NF-κB signaling through the inhibition of inhibitor of κB (IκB)α phosphorylation and upstream mitogen-activated protein kinase signaling (36). Notably, the action of ECL as an NF-κB inhibitor has been established using an NF-κB-driven luciferase reporter gene assay in TNF-α-activated 293/NF-κB-luc cells (22). These

findings prompted us to hypothesize that the anticancer activity of ECL is likely a result of the inhibition of NF- κ B, as well as its upstream signal transduction pathway, the AKT signaling pathway. The present study is the first to the best of our knowledge, to reveal the anticancer mechanism of ECL in the NSCLC A549 and Calu-1 cells via the induction of cell cycle arrest and cell apoptosis. Moreover, ECL was found to cause the upregulation of pro-apoptotic (cleaved) caspase-3 and cleaved PARP, as well as the downregulation of the expression of anti-apoptotic Bcl-xL and survivin proteins. As anticipated, ECL could also inhibit AKT and NF- κ B signaling in NSCLC cells.

The activation of the AKT pathway is frequently dysregulated in several types of cancer, including lung cancer, and is an important factor in the growth, survival and chemotherapeutic resistance of cancer cells (37). Increased AKT activation in human cancers can result from constitutive phosphorylation of AKT protein at the Ser473 site, due to aberrant PI3K activation (30). One of the important downstream signaling targets of AKT is NF- κ B. AKT controls the activity of NF- κ B via the phosphorylation of I κ B kinase (IKK) and subsequent degradation of the I κ B, which results in the release and translocation of NF- κ B into the nucleus (38). NF- κ B is a transcription factor that regulates the expression of numerous genes that are critical for the survival or inhibition of apoptotic cell death (39). Moreover, AKT/NF- κ B is one of the most important signaling pathways that promote lung carcinogenesis and regulate the inactivation of apoptosis in lung cancer (40,41). Therefore, AKT/NF- κ B signaling-induced apoptosis is a suitable target for anticancer therapy.

Suppression of AKT activity by specific synthetic inhibitors of PI3K such as wortmannin and LY294002 has been revealed to exert antitumor activity against several human solid tumor models (42). Notably, several natural products, such as epigallocatechin gallate (EGCG) and safflower polysaccharide (SPS) inhibited AKT and induced apoptosis in NSCLC cells (43,44). EGCG significantly reduced the level of phosphorylated-AKT, but the same treatment did not affect the levels of total AKT expression (43). Conversely, SPS inhibited both the expression of total and phosphorylated AKT (44). The present data demonstrated the inhibition of AKT by ECL. A significant decrease of p^(S473)-AKT/AKT ratio by ECL confirmed that ECL could suppress the AKT activation by inhibiting the phosphorylation of AKT at Serine473 (S473), which is the regulatory site responsible for its activity (30). Moreover, the p^(S536)-NF- κ B p65/NF- κ B p65 ratio was significantly reduced by ECL suggesting the inactivation of NF- κ B by ECL by suppressing the phosphorylation at the serine 536 (S536) position of the NF- κ B p65 subunit, which is required for the activation and nuclear translocation of NF- κ B (31). Since the suppression of AKT activation by an inhibitor of PI3K (LY294002) inhibited the phosphorylation of S536-NF- κ B p65, NF- κ B activation via phosphorylated S536 NF- κ B p65 is largely relied on AKT (45). Various natural agents such as piperlongumine (46) and oenothien B (4) have also been revealed to inhibit lung tumor growth and/or induce NSCLC cell apoptosis by blocking the NF- κ B activation. The suppression of NF- κ B activation by ECL is likely modulated through the inhibition of its upstream signaling, AKT kinase.

The AKT/NF- κ B signaling pathway is influential in the regulation of cell survival, due to the activation of anti-apoptotic

downstream effectors. The pro-apoptotic potential of some anticancer agents is highly correlated with the inactivation of the AKT/NF- κ B pathway (10,47). The NF- κ B-regulated gene products include anti-apoptotic and cell survival genes, such as Bcl-xL and survivin (48). Since ECL induces apoptosis in the NSCLC cells, this effect is associated with the inhibition of anti-apoptotic proteins, Bcl-xL and survivin, whose expression is controlled by NF- κ B. Subsequently, the high activation of pro-apoptotic caspase-3 and PARP occurred following treatment with ECL. The downregulation of anti-apoptotic proteins in conjunction with the upregulation of pro-apoptotic proteins in both A549 and Calu-1 cells treated with ECL likely serve to shift the balance from pro-survival to proapoptotic signaling. Therefore, ECL may induce the NSCLC cell apoptosis by inhibiting the AKT/NF- κ B activation.

An alternative mode of action of ECL has been proposed as an inhibitor of protein synthesis. A study in TNF- α -activated human endothelial cells revealed that ECL, rather than inhibiting the early NF- κ B signaling, post-transcriptionally downregulated the expression of the NF- κ B-dependent target genes, including ICAM-1, VCAM-1 and E-selectin. Notably, ECL alone could not inhibit the expression of survivin in endothelial cells (23). Not only the activation of AKT and NF- κ B, but also the expression of survivin, were found to be higher in cancer cells than in normal cells (6,49). Thus, ECL may effectively exert different modes of actions depending on the differences in cell types and differentiation.

Cisplatin is one of the most widely used chemotherapeutic drugs in the treatment of lung cancer (50). It is a DNA-damaging agent, which can covalently bind to DNA leading to different types of DNA lesions. Subsequently, cisplatin-DNA adducts cause various cellular responses, such as replication arrest, transcription inhibition, cell-cycle arrest, DNA repair, and apoptotic cell death (51). However, the efficacy of cisplatin in NSCLC treatment is limited due to either intrinsic or acquired cisplatin resistance. Several mechanisms have been proposed to account for the resistance of NSCLC tumor cells to cisplatin, including the disruption of apoptotic cell death pathways (52). In addition, a high AKT/NF- κ B activity in the NSCLC cells has been revealed to be highly associated with the apoptosis inhibition-related cisplatin resistance (53,54). Notably, cisplatin treatment can activate the AKT/NF- κ B signaling pathway in NSCLC cells, resulting in an anti-apoptotic effect that may also counteract the cisplatin cytotoxicity and lead to the development of cisplatin resistance (11). Moreover, IKK phosphorylation of NF- κ B at serine 536 could contribute to acquired cisplatin resistance (55). Therefore, inhibition of NF- κ B signaling favorably serves as a critical target for enhancing the efficacy of cisplatin in NSCLC treatment. The inhibition of the AKT/NF- κ B pathway by plant-derived compounds, such as baicalein (15) and tunicamycin (16) could enhance the cisplatin sensitivity of NSCLC cells. Significantly, the results of the present study demonstrated for the first time that ECL is in fact capable of sensitizing the NSCLC cells to cisplatin-mediated apoptosis, which is evidenced by the activation of caspase-3 as well as the downregulation of anti-apoptotic Bcl-xL and survivin proteins. Furthermore, cisplatin mediated the activation of AKT and NF- κ B phosphorylation in both NSCLC cell lines, while ECL blocked the AKT activation and almost completely inhibited the NF- κ B activation induced by

cisplatin. Likewise, two of the anti-apoptosis-related targets of NF- κ B, survivin and Bcl-xL, were further downregulated by combination treatment with cisplatin and ECL, as compared to treatment with cisplatin alone. Since cisplatin is still widely used as a chemotherapeutic drug, the long-term treatment of cisplatin causes cancer cells to develop chemoresistance by various mechanisms, including the activation of AKT/NF- κ B signaling (11). Therefore, ECL not only enhanced chemosensitivity, but may also further reduce the chemoresistance of NSCLC cells to cisplatin.

In conclusion, the present *in vitro* study has provided evidence of an underlying anticancer mechanism of ECL in NSCLC cells, through which ECL inactivates the AKT/NF- κ B-signaling pathway, leading to the induction of cancer cell apoptosis and chemosensitization to cisplatin. The insight gained from the present study indicated the potential application of ECL for potentiating cisplatin chemosensitivity for the effective treatment of NSCLC. Additional *in vivo* studies are required to confirm the result of ECL in combination with cisplatin, before entering this combination into a clinical trial that may offer a novel treatment option for patients with NSCLC.

Acknowledgements

The authors wish to acknowledge the Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand for providing research facilities.

Funding

The present study was supported by the Royal Golden Jubilee PhD (RGJ-PHD) Programme, Thailand Science Research and Innovation (TSRI) (grant no. PHD/0088/2558), the Faculty of Medicine Research Fund (grant no. 090-2560), Faculty of Medicine, Chiang Mai University, and a grant from the Faculty of Dentistry, Mahidol University, Thailand.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

ND, KC, PP and AW contributed to the conception, design and follow-up of the study. ND, KC and JK contributed to the acquisition, analysis, and interpretation of data. ND performed the statistical analysis and wrote the manuscript. AI and WT participated in the interpretation of data and provided critical revisions to the scientific content of the manuscript. PP and AW reviewed and edited the manuscript, and AW supervised the project. All the authors approved the final version of the manuscript and agree to be accountable for all aspects of this work in ensuring that questions related to the accuracy or integrity of any part of this work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Siegel RL, Miller KD and Jemal A: Cancer statistics, 2018. *CA Cancer J Clin* 68: 7-30, 2018.
2. Lilloglou T, Bediaga NG, Brown BR, Field JK and Davies MP: Epigenetic biomarkers in lung cancer. *Cancer Lett* 342: 200-212, 2014.
3. D'Addario G, Pintilie M, Leighl NB, Feld R, Cerny T and Shepherd FA: Platinum-based versus non-platinum-based chemotherapy in advanced non-small-cell lung cancer: A meta-analysis of the published literature. *J Clin Oncol* 23: 2926-2936, 2005.
4. Pei X, Xiao J, Wei G, Zhang Y, Lin F, Xiong Z, Lu L, Wang X, Pang G, Jiang Y and Jiang L: Oenothien B inhibits human non-small cell lung cancer A549 cell proliferation by ROS-mediated PI3K/Akt/NF- κ B signaling pathway. *Chem Biol Interact* 298: 112-120, 2019.
5. Hafsi S, Pezzino FM, Candido S, Ligresti G, Spandidos DA, Souza Z, McCubrey JA, Travali S and Libra M: Gene alterations in the PI3K/PTEN/AKT pathway as a mechanism of drug-resistance (review). *Int J Oncol* 40: 639-644, 2012.
6. Hakan Kucuksayan H, Sakir Akgun S and Akca H: PI3K/Akt/NF- κ B signalling pathway on NSCLC invasion. *Med Chem* 6: 4, 2016.
7. Naugler WE and Karin M: NF- κ B and cancer-identifying targets and mechanisms. *Curr Opin Genet Dev* 18: 19-26, 2008.
8. Chen W, Liu X, Yuan S and Qiao T: HSPA12B overexpression induces cisplatin resistance in non-small-cell lung cancer by regulating the PI3K/Akt/NF- κ B signaling pathway. *Oncol Lett* 15: 3883-3889, 2018.
9. Zou W, Ma X, Hua W, Chen B and Cai G: Caveolin-1 mediates chemoresistance in cisplatin-resistant ovarian cancer cells by targeting apoptosis through the Notch-1/Akt/NF- κ B pathway. *Oncol Rep* 34: 3256-3263, 2015.
10. Godwin P, Baird AM, Heavey S, Barr MP, O'Byrne KJ and Gately K: Targeting nuclear factor-kappa B to overcome resistance to chemotherapy. *Front Oncol* 3: 120, 2013.
11. Albanell J, Chattopadhyay S, Tapia M, Rovira A, Belda-Iniesta C, Manguán-García JDC, Machado R, Gonzalez Barón M and Perona R: JNK/MKP-1 and PI3K/NF- κ B: Critical pathways controlling cellular response towards cisplatin in non-small cell lung cancer (NSCLC). *J Clin Oncol* 23: 2033-2033, 2005.
12. Nobili S, Lippi D, Witort E, Donnini M, Bausi L, Mini E and Capaccioli S: Natural compounds for cancer treatment and prevention. *Pharmacol Res* 59: 365-378, 2009.
13. Li Y, Ahmed F, Ali S, Philip PA, Kucuk O and Sarkar FH: Inactivation of nuclear factor kappaB by soy isoflavone genistein contributes to increased apoptosis induced by chemotherapeutic agents in human cancer cells. *Cancer Res* 65: 6934-6942, 2005.
14. Liu D, Yan L, Wang L, Tai W, Wang W and Yang C: Genistein enhances the effect of cisplatin on the inhibition of non-small cell lung cancer A549 cell growth in vitro and in vivo. *Oncol Lett* 8: 2806-2810, 2014.
15. Yu M, Qi B, Wu X, Xu J and Liu X: Baicalein increases cisplatin sensitivity of A549 lung adenocarcinoma cells via PI3K/Akt/NF- κ B pathway. *Biomed Pharmacother* 90: 677-685, 2017.
16. Ahmmed B, Khan MN, Nisar MA, Kampo S, Zheng Q, Li Y and Yan Q: Tunicamycin enhances the suppressive effects of cisplatin on lung cancer growth through PTX3 glycosylation via AKT/NF- κ B signaling pathway. *Int J Oncol* 54: 431-442, 2019.
17. Miyake K, Tezuka Y, Awale S, Li F and Kadota S: Quassinoids from *Eurycoma longifolia*. *J Nat Prod* 72: 2135-2140, 2009.
18. Rehman SU, Choe K and Yoo HH: Review on a traditional herbal medicine, *Eurycoma longifolia* Jack (tongkat ali): Its traditional uses, chemistry, evidence-based pharmacology and toxicology. *Molecules* 21: 331, 2016.
19. Kuo PC, Damu AG, Lee KH and Wu TS: Cytotoxic and antimarial constituents from the roots of *Eurycoma longifolia*. *Bioorg Med Chem* 12: 537-544, 2004.

20. Miyake K, Li F, Tezuka Y, Awale S and Kadota S: Cytotoxic activity of quassinoids from *Eurycoma longifolia*. *Nat Prod Commun* 5: 1009-1012, 2010.
21. Dukaew N, Konishi T, Chairatvit K, Autsavapromporn N, Soonthorncharenonn N and Wongnoppavich A: Enhancement of radiosensitivity by eurycomalactone in human NSCLC cells through G2/M cell cycle arrest and delayed DNA double-strand break repair. *Oncol Res* 28: 161-175, 2020.
22. Tran TV, Malainer C, Schwaiger S, Atanasov AG, Heiss EH, Dirsch VM and Stuppner H: NF- κ B inhibitors from *Eurycoma longifolia*. *J Nat Prod* 77: 483-488, 2014.
23. Malainer C, Schachner D, Sangiovanni E, Atanasov AG, Schwaiger S, Stuppner H, Heiss EH and Dirsch VM: Eurycomalactone inhibits expression of endothelial adhesion molecules at a post-transcriptional level. *J Nat Prod* 80: 3186-3193, 2017.
24. Phannasorn W, Khanaree C, Wongnoppavich A and Chewonarin T: The effect of purple rice (*Oryza sativa* L. indica) extract on the inflammatory response in a colon cancer cell line and dextran sulfate-induced tumor promotion in the rat colon. *Mol Cell Toxicol* 13: 433-442, 2017.
25. Peters GJ, van der Wilt CL, van Moorsel CJ, Kroep JR, Bergman AM and Ackland SP: Basis for effective combination cancer chemotherapy with antimetabolites. *Pharmacol Ther* 87: 227-253, 2000.
26. Chou TC: Drug combination studies and their synergy quantification using the Chou-Talalay method. *Cancer Res* 70: 440-446, 2010.
27. Wongnoppavich A, Dukaew N, Choonate S and Chairatvit K: Upregulation of maspin expression in human cervical carcinoma cells by transforming growth factor beta1 through the convergence of Smad and non-Smad signaling pathways. *Oncol Lett* 13: 3646-3652, 2017.
28. Kaltschmidt C, Banz-Jansen C, Benhidjeb T, Beshay M, Förster C, Greiner J, Hamelmann E, Jorch N, Mertzlufft F, Pfitzenmaier J, *et al*: A role for NF- κ B in organ specific cancer and cancer stem cells. *Cancers (Basel)* 11: 655, 2019.
29. Huang WC and Hung MC: Induction of Akt activity by chemotherapy confers acquired resistance. *J Formos Med Assoc* 108: 180-194, 2009.
30. Liao Y and Hung MC: Physiological regulation of Akt activity and stability. *Am J Transl Res* 2: 19-42, 2010.
31. Hu J, Nakano H, Sakurai H and Colburn NH: Insufficient p65 phosphorylation at S536 specifically contributes to the lack of NF- κ B activation and transformation in resistant JB6 cells. *Carcinogenesis* 25: 1991-2003, 2004.
32. Barta JA, Powell CA and Wisnivesky JP: Global epidemiology of lung cancer. *Ann Glob Health* 85: 8, 2019.
33. Ijaz S, Akhtar N, Khan MS, Hameed A, Irfan M, Arshad MA, Ali S and Asrar M: Plant derived anticancer agents: A green approach towards skin cancers. *Biomed Pharmacother* 103: 1643-1651, 2018.
34. Wong PF, Cheong WF, Shu MH, Teh CH, Chan KL and AbuBakar S: Eurycomanone suppresses expression of lung cancer cell tumor markers, prohibitin, annexin 1 and endoplasmic reticulum protein 28. *Phytomedicine* 19: 138-144, 2012.
35. Zakaria Y, Rahmat A, Pihie AH, Abdullah NR and Houghton PJ: Eurycomanone induce apoptosis in HepG2 cells via up-regulation of p53. *Cancer Cell Int* 9: 16, 2009.
36. Hajjoui S, Chateauvieux S, Teiten MH, Orlikova B, Schumacher M, Dicato M, Choo CY and Diederich M: Eurycomanone and eurycomanol from *Eurycoma longifolia* Jack as regulators of signaling pathways involved in proliferation, cell death and inflammation. *Molecules* 19: 14649-14666, 2014.
37. Tokunaga E, Oki E, Egashira A, Sadanaga N, Morita M, Kakeji Y and Maehara Y: Deregulation of the Akt pathway in human cancer. *Curr Cancer Drug Targets* 8: 27-36, 2008.
38. Bai D, Ueno L and Vogt PK: Akt-mediated regulation of NF κ B and the essentialness of NF κ B for the oncogenicity of PI3K and Akt. *Int J Cancer* 125: 2863-2870, 2009.
39. Oeckinghaus A and Ghosh S: The NF- κ B family of transcription factors and its regulation. *Cold Spring Harb Perspect Biol* 1: a000034, 2009.
40. Chen W, Li Z, Bai L and Lin Y: NF- κ B in lung cancer, a carcinogenesis mediator and a prevention and therapy target. *Front Biosci (Landmark Ed)* 16: 1172-1185, 2011.
41. Umemura S, Mimaki S, Makinoshima H, Tada S, Ishii G, Ohmatsu H, Niho S, Yoh K, Matsumoto S, Takahashi A, *et al*: Therapeutic priority of the PI3K/AKT/mTOR pathway in small cell lung cancers as revealed by a comprehensive genomic analysis. *J Thorac Oncol* 9: 1324-1331, 2014.
42. Garcia-Echeverria C and Sellers WR: Drug discovery approaches targeting the PI3K/Akt pathway in cancer. *Oncogene* 27: 5511-5526, 2008.
43. Gu JJ, Qiao KS, Sun P, Chen P and Li Q: Study of EGCG induced apoptosis in lung cancer cells by inhibiting PI3K/Akt signaling pathway. *Eur Rev Med Pharmacol Sci* 22: 4557-4563, 2018.
44. Li JY, Yu J, Du XS, Zhang HM, Wang B, Guo H, Bai J, Wang JH, Liu A and Wang YL: Safflower polysaccharide induces NSCLC cell apoptosis by inhibition of the Akt pathway. *Oncol Rep* 36: 147-154, 2016.
45. Kwon HJ, Choi GE, Ryu S, Kwon SJ, Kim SC, Booth C, Nichols KE and Kim HS: Stepwise phosphorylation of p65 promotes NF- κ B activation and NK cell responses during target cell recognition. *Nat Commun* 7: 11686, 2016.
46. Zheng J, Son DJ, Gu SM, Woo JR, Ham YW, Lee HP, Kim WJ, Jung JK and Hong JT: Piperlongumine inhibits lung tumor growth via inhibition of nuclear factor kappa B signaling pathway. *Sci Rep* 6: 26357, 2016.
47. Khan KH, Yap TA, Yan L and Cunningham D: Targeting the PI3K-AKT-mTOR signaling network in cancer. *Chin J Cancer* 32: 253-265, 2013.
48. Shukla S, Shankar E, Fu P, MacLennan GT and Gupta S: Suppression of NF- κ B and NF- κ B-regulated gene expression by apigenin through I κ B α and IKK pathway in TRAMP mice. *PLoS One* 10: e0138710, 2015.
49. Cheung CH, Huang CC, Tsai FY, Lee JY, Cheng SM, Chang YC, Huang YC, Chen SH and Chang JY: Survivin-biology and potential as a therapeutic target in oncology. *Onco Targets Ther* 6: 1453-1462, 2013.
50. Rinaldi M, Cauchi C and Gridelli C: First line chemotherapy in advanced or metastatic NSCLC. *Ann Oncol* 17 (Suppl 5): v64-v67, 2006.
51. Galluzzi L, Senovilla L, Vitale I, Michels J, Martins I, Kepp O, Castedo M and Kroemer G: Molecular mechanisms of cisplatin resistance. *Oncogene* 31: 1869-1883, 2012.
52. Köberle B, Tomicic MT, Usanova S and Kaina B: Cisplatin resistance: Preclinical findings and clinical implications. *Biochim Biophys Acta* 1806: 172-182, 2010.
53. Baby J, Pickering BF, Vashisht Gopal YN and Van Dyke MW: Constitutive and inducible nuclear factor-kappaB in immortalized normal human bronchial epithelial and non-small cell lung cancer cell lines. *Cancer Lett* 255: 85-94, 2007.
54. Chen B, Shen Z, Wu D, Xie X, Xu X, Lv L, Dai H, Chen J and Gan X: Glutathione peroxidase 1 promotes NSCLC resistance to cisplatin via ROS-induced activation of PI3K/AKT pathway. *Biomed Res Int* 2019: 7640547, 2019.
55. Li Z, Yang Z, Lapidus RG, Liu X, Cullen KJ and Dan HC: IKK phosphorylation of NF- κ B at serine 536 contributes to acquired cisplatin resistance in head and neck squamous cell cancer. *Am J Cancer Res* 5: 3098-3110, 2015.



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