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Amentoflavone Induces Cell-cycle Arrest, Apoptosis, and Invasion Inhibition in Non-small Cell Lung Cancer Cells

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Abstract. *Background/Aim: Amentoflavone, an effective compound derived from medicinal plants, has been shown to boost therapeutic efficacy of chemotherapy in non-small cell lung cancer (NSCLC). However, anti-NSCLC effect of amentoflavone is ambiguous. The major purpose of the present study was to verify the inhibitory effects of amentoflavone in NSCLC cells. Materials and Methods: The effects of amentoflavone on growth and invasion of NSCLC CL-1-5-F4 cells were evaluated by cell viability assay, flow cytometry, colony formation assay, nuclear factor-kappa B (NF-κB) reporter gene assay, immunofluorescence staining, transwell invasion, and western blot assay. Results: Amentoflavone effectively induced cell growth inhibition, G1 cell-cycle arrest, apoptosis, and suppression of invasion. Furthermore, amentoflavone not only triggered expression of p27, cleaved caspase-3, -8 also reduced NF-κB signaling, protein levels of matrix metalloproteinase (MMP)-2, -9, Cyclin-D1, and vascular endothelial growth factor (VEGF). Conclusion: Cell-cycle arrest, apoptosis induction, NF-κB signaling inhibition are associated with amentoflavone-inhibited growth and invasion of NSCLC cells.*

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Non-small cell lung cancer (NSCLC), the most common form of lung cancer, is divided into three morphological subtypes: adenocarcinoma, squamous cell carcinoma (SCC), and large-cell carcinoma (LCC) (1). The survival rate of patients with NSCLC is prolonged by current treatment strategies including surgery, radiation, chemotherapy, targeted therapy, and immunotherapy (1-3). Herbal medicines have been recognized as a complementary therapy to reduce the side-effects of chemotherapy and improve prognosis of patients with NSCLC (4-6).

Herbal products or compounds isolated from herbal medicines have been shown to eliminate tumor growth and invasion ability through induction of apoptosis, cell-cycle arrest, and blockage of invasion-related signaling pathways in NSCLC cells (1, 7, 8). Bruceine D, the bioactive compound extracted from medicinal plant Brucea javanica's fruit, induced apoptosis *via* Jun N-terminal kinase (JNK)-mediated intrinsic pathway in NSCLC cells (9). Berberine, the anticancer compound derived from Chinese herbs, elicited cell cycle arrest and attenuated invasion of NSCLC cells by decreasing 3-phosphoinositide-dependent protein kinase-1 (PDPK1) and transcription factor SP1 protein expressions (10).

Amentoflavone, a bioactive ingredient derived from medicinal plants, has been demonstrated to possess biological properties such as anti-inflammatory, anti-cancer, neuroprotective, and

cardioprotective activities (11). Previous studies showed that amentoflavone inhibited tumor cell growth and invasion in hepatocellular carcinoma, glioblastoma, and bladder cancer (12-14). Amentoflavone has been shown as poly (ADP-ribose) polymerase-1 (PARP-1) inhibitor to enhance therapeutic efficacy of carboplatin in NSCLC *in vitro* and *in vivo* (15). However, anti-NSCLC effect of amentoflavone has not yet been elucidated. Therefore, the main purpose of the present study was to evaluate the inhibitory effect of amentoflavone on tumor cell growth and invasion in NSCLC cells.

Materials and Methods

Chemicals, antibodies and reagents. Amentoflavone was purchased from ChemFaces (Wuhan, China) and dissolved in DMSO to 100 mM. Hygromycin B and D-luciferin were obtained from MedChemExpress (Monmouth Junction, NJ, USA) and Promega (Madison, WI, USA), respectively. 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT), dimethyl sulfoxide (DMSO), and crystal violet were all purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12), fetal bovine serum (FBS), and penicillin-streptomycin (P.S) were purchased from GIBCO®/Invitrogen Life Technologies (Carlsbad, CA, USA). Primary antibodies against NF-κB p65 (E-AB-22066, rabbit, Elabscience Biotechnology Inc), matrix metalloproteinase-9 (MMP-9) (AB19016, rabbit, EMD Millipore, Billerica, MA, USA), matrix metalloproteinase-2 (MMP-2) (10373-2-AP, rabbit, Proteintech, Rosemont, IL, USA), vascular endothelial growth factor (VEGF) (ab1316, mouse, Abcam, Cambridge, UK), myeloid cell leukemia-1 (MCL-1) (BV-438, rabbit, BioVision, Milpitas, CA, USA), cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein (C-FLIP) (D16A8, rabbit, Cell signaling Technology Danvers, MA, USA), X-linked inhibitor of apoptosis protein (XIAP) (PA5-29253, rabbit, Thermo Fisher Scientific, Waltam, MA, USA), β-actin (sc-47778, mouse, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for western blotting were purchased from different companies as listed. HRP-conjugated secondary antibodies and Alexa Fluor 488-conjugated secondary antibodies, Peroxidase AffiniPure Goat Anti-Mouse IgG and Goat Anti-Rabbit IgG were purchased from Jackson ImmunoResearch (West Grove, PA, USA).

Cell culture. Non-small cell lung cancer CL1-5-F4 cells were provided by Dr. Chia-Lin Hsieh (Taipei Medical University, Taiwan, ROC). CL1-5-F4 cells were cultured with DMEM/F-12 containing 10% fetal bovine serum and 1% PS (10,000 units/ml of penicillin and 10,000 μg/ml of streptomycin). The cells were incubated at 95% air and 5% CO_2 gas mixture under 37˚C and at humidified environment.

Plasmid transfection, stable clone selection and reporter gene assay. CL1-5-F4 cells were transfected with pGL4/*NF-κB/luc2* (Promega, Madison, WI, USA) vector using JetPEI™ transfection kit (Illkirch, Bas-Rhin, France). CL1-5-F4 cells with the functional of NF-κB reporter gene and luciferase expressed signal was renamed as CL1-5-F4/*NF-κB/luc2* cells. Detail procedure of plasmid transfection and reporter gene assay were described in previous study (16).

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay. Cell viability was performed by the MTT assay. The 5 mg/ml concentration of MTT stock was prepared in phosphate-buffered saline (PBS) before use. CL1-5-F4 cells were seeded into 96-well plates at a density of 5×10^3 cells/well and incubated overnight. Cells were treated with various concentrations of amentoflavone (0~350 μM) for 24 and 48 h. After treatment, we discarded the supernatant, replaced with MTT solution and incubated at 37˚C in dark for 4 h. Before optical intensity (Molecular Devices, San Jose, CA, USA) detection, MTT solution was replaced with 100 μl DMSO. The absorbance wavelength of MTT is 570 nm, the blank value was defined as zero (+/–0.1) (17).

Flow cytometry analyzes. CL1-5-F4 cells were seeded in 6 well plates (1×10^5) and treated with 0, 75 and 150 μM amentoflavone for 48 h. For cell-cycle assay, cells were permlized by 70% ethanol in -20˚C overnight. Then, cells were stained with propidium iodide at 37˚C in dark. For cleaved caspase-3, and -8 staining, cells were added 100 μl wash buffer containing 1 μl cleaved caspase-3 or 8 FITC-antibodies (CaspGLOW™ Fluorescence Active Caspase-3 or 8 Staining Kit, BioVision) and incubated in 37˚C in dark. For AnnexinV/PI assay, cells were stained with FITC annexinV apoptosis detection kit I (BD, Cat NO. 556547). For cleaved PARP-1 staining, cells were fixed with 4% paraformaldehyde, permeabilized with 90% methanol on ice 30 min and followed with FITC-cleaved PARP-1 antibody staining for 1 h. After different kinds of cells staining process, the fluorescence intensities released from cells were than evaluated by flow cytometry (NovoCyto®, ACEA Bioscience, Inc., USA). Finally, the fluorescence intensity change of each group was analysis and quantified by NovoExpress® software.

Clonogenic formation assay. CL1-5-F4 cells were seeded in 6 well plates (1×10^5) and treated with 0, 75 and 150 μM of amentoflavone for 48 h. Then, 1,000 cells, 1,500 cells, and 2,000 cells were harvested from 0, 75 and 150 μM amentoflavone treated group, respectively, and seeded into 6 cm plate for 2 weeks' incubation. After that, the clones were fixed with fixation buffer (Acetic acid: Methanol=1: 3) and stained with 1% crystal violet. The colony is defined to consist of at least 50 cells. The surviving fraction (SF) was defined as the colonies counted divided by the number of cells plated after plating efficiency (PE) correction (18). [PE=colonies observed ÷ number of cells plated; SF=colonies counted ÷ (cells seeded × PE)].

NF-κB translocation assay. CL1-5-F4 cells were seeded into 4 wells chamber slides (5×10^4) overnight and treated with 0, 75 and 150 μM amentoflavone for 48 h. After 48 h treatment, cells were fixed with 4% paraformaldehyde in PBS for 15 min and permeabilized with 0.1% Triton X-100/PBS for 10 min. The slides were rinsed three times with PBS, blocking with 1% bovine serum albumin (BSA)/PBS for 1 h. Then, primary antibody (1:300) was added on slides overnight at 4˚C. The slides were rinsed three times with PBST (0.01% Tween 20), Alexa Fluor®488 conjugated-secondary goat-anti rabbit antibody was added on slides for 1 h protected from light. Rinsed 3 times with PBST, we mounted slides with DAPI containing mounting buffer. Finally, observed images with fluorescence microscopy (Axio Imager 2, Zeiss, NY, USA).

Trans-well invasion assay. CL1-5-F4 cells were pre-treated with 0, 75 and 150 μM of amentoflavone. Cells were then harvested at 5×10^4 cells from each group, seeded onto 8 μm pore size transwells (with matrigel) and incubated for another 24 h. After 24 h, transwells were fixed with fixation buffer (Acetic acid: Methanol=1: 3) and stained with 1% crystal violet. Tranwell membranes were finally observed by light microscopy. Detail procedure of trans-well assay was described in previous study (19).

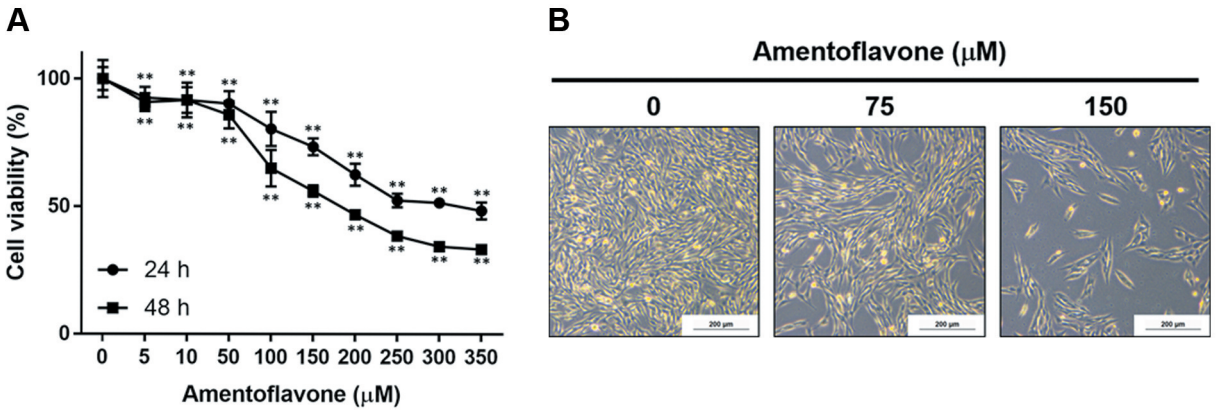

Figure 1. *Cell viability was inhibited by amentoflavone in CL1-5-F4 cells. (A) CL1-5-F4 cells are treated with 0 to 350 μM amentoflavone for 24 and 48 h and cell viability is tested with the MTT assay. (B) Representative light microscopy images of CL1-5-F4 cells from each group after 0, 75 and 150 μM amentoflavone treatment are displayed. **p<0.01 vs. 0 μM amentoflavone. Scale bars: 200 μm.*

Western blotting. CL1-5-F4 cells were harvested and lysed with NP40 lysis buffer (Containing proteinase and phosphatase inhibitors) to collect total proteins. Proteins were separated with 6% to 12% SDS-PAGE gels, and transferred on PVDF membranes (EMD Millipore, Bedford, Massachusetts). Then, the membranes were blocking with 5% non-fat milk for 1 h. The primary and HRP-conjugated secondary antibodies were added separately. The procedure details were described in previous study (20).

Statistical analysis. We used Excel 2019 to perform statistical analysis of our results. Student's *t*-test was used to calculate the statistical difference between untreated and treated groups. All statistical results are presented as means±standard error. *p*-Values less than 0.05 were defined as significance difference between untreated and treated groups.

Results

Amentoflavone inhibited the viability of NSCLC CL1-5-F4 cells. To evaluate the cytotoxicity of amentoflavone in the NSCLC cell line CL1-5-F4. We used MTT assay to test the cytotoxicity of amentoflavone from 0 to 350 μM in CL1-5-F4 cells for 24 and 48 h (Figure 1A). MTT assay showed a reduction of CL1-5-F4 cell viability after amentoflavone treatment in dose-dependent manner and time-dependent manner. In the meantime, the morphology of cells after three different amentoflavone treatment dosages (0, 75, 150 μM) were observed by light microscopy (Figure 1B). The densities of cells were markedly decreased in 75 μM and 150 μM of amentoflavone treatment groups. In sum, amentoflavone induces cytotoxicity of CL1-5-F4 cells.

Amentoflavone may only slightly induce apoptosis in CL1-5-F4 cells. To further investigate whether amentoflavone could induce apoptosis of CL1-5-F4 cells, we evaluated the expression of several apoptosis markers by flow cytometry. As illustrated in AnnexinV/PI staining and cleaved caspase-3 staining in CL1-5-F4 cells after amentoflavone treatment (Figure 2A and B), the activation of AnnexinV/PI and cleaved caspase-3 were slightly increased. Cleaved caspase-3 staining results, showed a significant difference in the amentoflavone 150 μM group compared to the 0 μM group. In addition, the activation of cleaved PARP-1 was also use to detect apoptosis effects in cells. The 75 and 150 μM amentoflavone treatment showed a slight induction of PARP-1 activation (Figure 2C). Then, we also investigated the activation of extrinsic apoptosis markers, cleaved caspase-8 by flow cytometry. In Figure 2D, the marked induction effect of cleaved caspase-8 was found in the amentoflavone-treated group, which indicated the activation of extrinsic apoptosis signaling. According to the above results, we suggested that amentoflavone may slightly induce apoptosis effect in CL1-5-F4 cells.

Amentoflavone suppressed proliferation and migration abilities in CL1-5-F4 cells. Although the apoptosis mechanism may not be dramatically activated by amentoflavone, we further investigated whether amentoflavone may suppress proliferation and migration of NSCLC. For proliferation analysis, we performed cell-cycle analysis (Figure 3A) and clonogenic assay in CL1-5-F4 cells after amentoflavone treatment. As indicated in Figure 3A-B, G1 phase cells were significantly increased in 75 and 150 μM of amentoflavone treatment (Figure 3B). Amentoflavone successfully induced G_1 arrest of CL1-5-F4 cells. However, the apoptotic population, sub-G_1 phase cells were not significantly increased after amentoflavone treatment (Figure 3C). Furthermore, in clonogenic assay, the surviving fraction was decreased in both 75 μM 150 μM of amentoflavone treatment with dose-dependent manner (Figure 3D). We further tested the expression of p27 and cyclin D1 proteins by western blotting to investigate the inhibition of

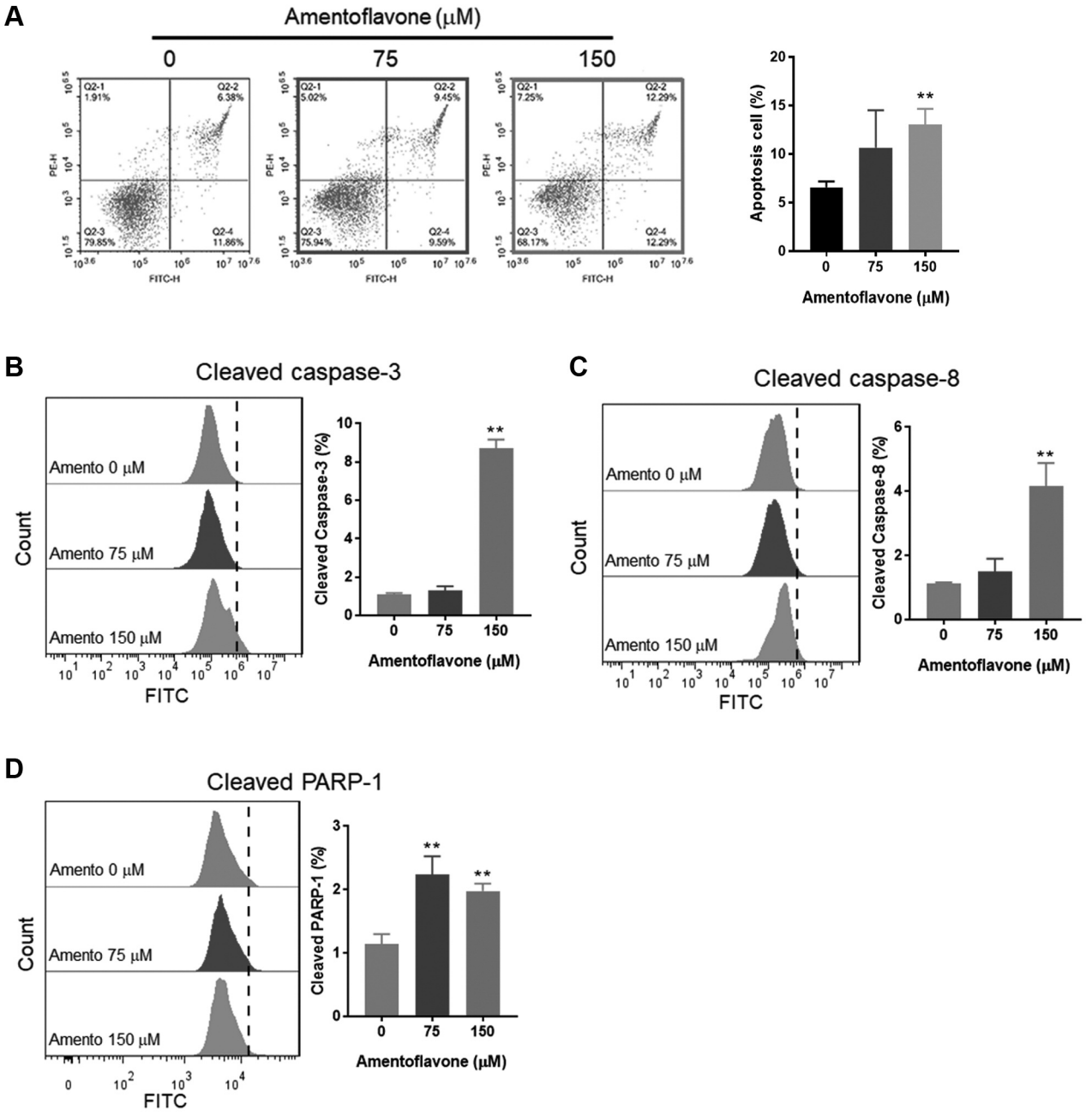

Figure 2. *The apoptosis effect was slightly induced by amentoflavone in CL1-5-F4 cells. CL1-5-F4 cells are treated with 0, 75 and 150 μM amentoflavone for 48 h, and tested by (A) AnnexinV/PI staining, (B) cleaved caspase-3 staining, (C) cleaved PARP-1 staining and (D) cleaved caspase-8 staining using flow cytometry. *p<0.05 vs. 0 μM amentoflavone; **p<0.01 vs. 0 μM amentoflavone.*

proliferation-related proteins expression. As shown in Figure 3E, the expression of cyclin D1 was decreased, in the contrary, the expression of p27 was increased. These results suggested that the toxicity of amentoflavone on NSCLC may not majorly contribute by apoptosis mechanism activation but affect by reducing cells proliferation in CL1-5-F4 cells.

Trans-well assay was then used to verify whether amentoflavone may suppress invasion ability in CL1-5-F4 cells. After amentoflavone treatment, the numbers of invading cells were significantly suppressed (Figure 3F). To further investigate invasion and proliferation ability associated proteins expression, the protein expression of MMP-2, -9, VEGFA and cyclin D1 were tested by western blotting. In Figure 3G, it is

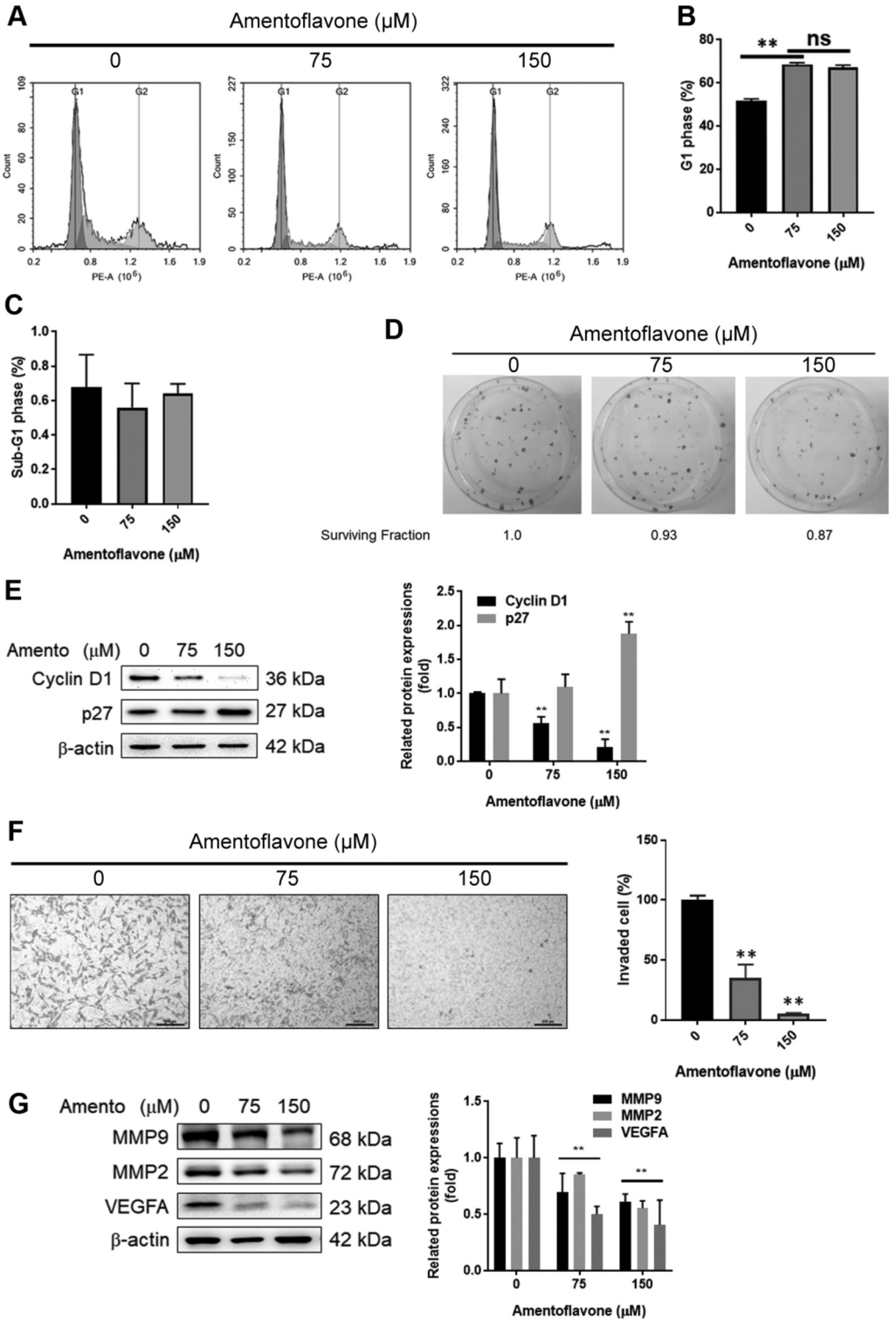

Figure 3. *The proliferation and invasion ability were decreased by amentoflavone treatment in CL1-5-F4 cells. CL1-5-F4 cells are treated with 0, 75 and 150 μM of amentoflavone for 48 h. (A) Cell-cycle assay was observed by flow cytometry, then (B) the population of G1 phase cells and (C) sub-G1 phase cells were quantified. (D) The colony of cells from each group is photographed and the surviving fraction is calculated. (E) The protein expression pattern and quantification results of p27 and cyclin D1 are displayed. (F) The photograph of transwell membranes and quantification of results are displayed. (G) Invasion and proliferation related proteins MMP2, 9 and VEGFA are tested by western blotting. **p<0.01 vs. 0 μM amentoflavone. Scale bars: 200 μm.*

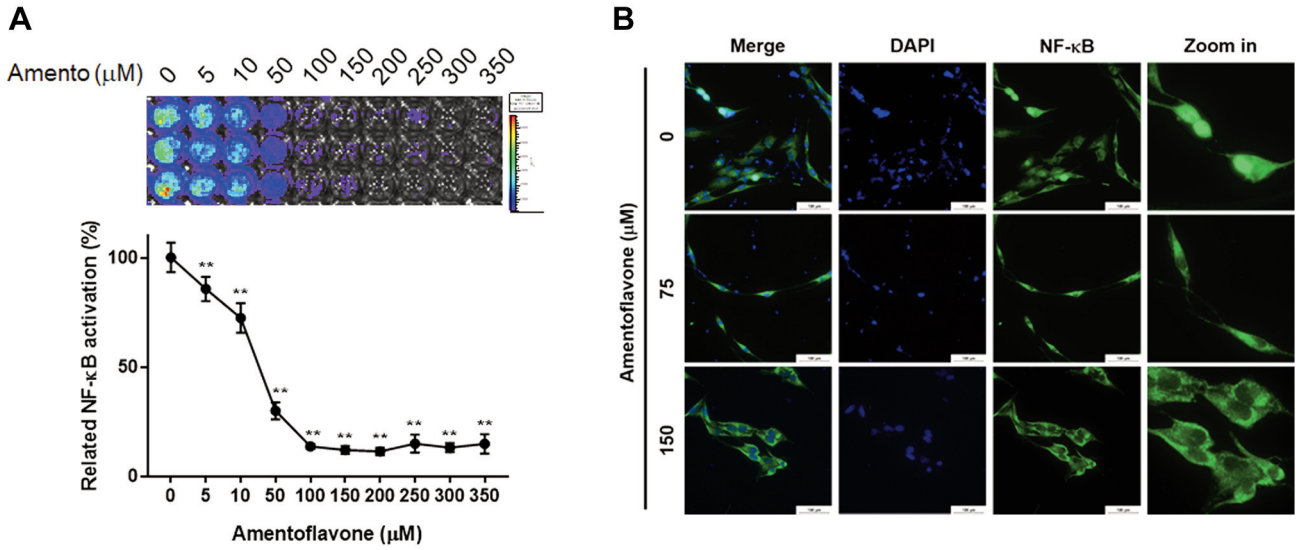

Figure 4. *The NF-κB activation and translocation were suppressed by amentoflavone treatment in CL1-5-F4 cells. (A) CL1-5-F4/NF-κB/luc2 cells were treated with 0 to 350 μM of amentoflavone for 48 h. Then, the NF-κB activation was evaluated by reporter gene assay using Xenogen IVIS spectrum instrument. (B) NF-κB translocation assay is also tested in CL1-5-F4 cells after 0, 75 and 150 μM of amentoflavone treatment. **p<0.01 vs. 0 μM amentoflavone. Scale bars: 100 μm.*

shown that amentoflavone may down-regulate protein expression of MMP-2, -9, VEGFA and cyclinD1. In sum, the proliferation and migration abilities of CL1-5-F4 cells were inhibited by amentoflavone.

Amentoflavone inhibited the activation of NF-κB in CL1-5-F4 cells. CL1-5-F4 cells were transfected with pGL4/*NF-κB/luc2* plasmid to establish a NF-κB reporter gene system. Transfected cells were renamed as CL1-5-F4/*NF-κB/luc2* cells. For NF-κB reporter gene assay, we treated different concentrations of amentoflavone (0-350 μM) in CL1-5-F4/*NF-κB/luc2* cells for 48 h. As showed in figure 4A, the activation of NF-κB was decreased by amentoflavone as dose-dependent manner.

NF-κB is a nuclear transcription factor which may need to translocate into the nucleus to active downstream gene expression. Here, NF-κB translocation assay was performed to identify the activation of NF-κB in CL1-5-F4 cells after amentoflavone treatment (Figure 4B). In 75 and 150 μM of amentoflavone treatment, more green fluorescence (NF-κB) signals were maintained in plasma instead of nuclear as compared to 0 μM of amentoflavone. These results indicated that amentoflavone may reduce the activation and nuclear translocation of NF-κB.

Discussion

Both inhibition of cell-cycle progression and induction of apoptosis down-regulate tumor cell growth (21). p27 (KIP1), a cyclin-dependent kinase (CDK) inhibitor, halts cell-cycle progression by preventing catalytic activity of cyclin-CDK complexes. Overexpression of p27 has been indicated to induce G_1 cell-cycle arrest (22, 23). Cleaved caspase-3, the key executioner of apoptosis, induces poly (ADP-ribose) polymerase-1 (PARP-1) cleavage and initiates apoptotic DNA fragmentation. The expression of cleaved caspase-3 is increased by upstream caspases such as (caspase-8 and -9) (24-26). The increased expression of p27 or caspase-3 was associated with favorable survival of patients with NSCLC (25, 27). Our results showed that amentoflavone significantly induced cell growth inhibition, G1 cell cycle arrest, and apoptosis in CL-1-5-F4 cells (Figures 1, 2 and 3A-B). Expression of p27, cleaved caspase-8, -3, and cleaved PARP-1 was significantly triggered by amentoflavone treatments (Figure 2B-D and 3E).

NF-κB, a heterodimeric transcription factor composed of p50 and p65 subunits, mediates tumor growth and invasion through regulating expression of proliferation and invasion-associated oncogenes such as cyclin-D1, MMP-9, MMP-2, and VEGF (28). The aberrant expression of NF-κB signaling was correlated not only with lymph node metastasis, but also with unfavorable survival in patients with NSCLC (29). Inhibition of NF-κB signaling has been demonstrated to eradicate protein levels of cyclin-D1, MMP-9, MMP-2, and VEGF in NSCLC cells (19, 30). Our results showed that amentoflavone blocked NF-κB signaling and nuclear translocation of NF-κB p65 (Figure 4). In addition, we also found that the expression of above-mentioned proliferation

and invasion-associated proteins was significantly diminished with amentoflavone treatments (Figure 3G). Blockage of Cyclin-D1 expression has been presented to induce G_1 cell-cycle arrest resulting in tumor growth inhibition (31). In addition to p27 overexpression, inhibition of cyclin-D1 expression was associated with amentoflavone-induced G1 cell-cycle arrest. Suppression of MMP-9, MMP-2, and VEGF expression has been shown to reduce invasion ability of NSCLC cells (32-35). The decreased expression of invasion-associated proteins was conducive to amentoflavone-inhibited invasion of NSCLC cells.

The strength of this study was that our results indicated the amentoflavone induced anti-NSCLC potential is associated with NF-κB inactivation. However, the therapeutic efficacy of amentoflavone was only performed on an *in vitro* system, further *in vivo* analyses need to be performed in the future. In conclusion, amentoflavone as a potential anticancer agent down-regulates cell growth and invasion in NSCLC cells. G1 cell-cycle arrest, induction of apoptosis, and suppression of NF-κB singling are associated with amentoflavone-inhibited growth and invasion of NSCLC cells.

Conflicts of Interest

The Authors declare no conflicts of interest with the contents of this article.

Authors' Contributions

Data curation, WT Chen, CH Chen, HT Su, PF Yueh, FT Hsu and IT Chiang; funding acquisition, WT Chen, CH Chen and HT Su; writing – original draft, FT Hsu and IT Chiang; writing – review, IT Chiang. All Authors have read and agreed to the published version of the manuscript.

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