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Original Article

Chelidonine inhibits melanoma cell malignancy by inactivating TLR4/NF-κB and PI3K/AKT signaling pathways

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ABSTRACT Melanoma is a common and aggressive tumor, characterized by a high incidence rate and extensive metastasis. Chelidonine exhibits a broad range of biological properties including anti-inflammatory, antimicrobial, and anticancer effects. Our study is intended to explore the effects chelidonine of on melanoma cells. In detail, CCK-8 assay was used for detection of cell viability. The colony formation assay was carried out to measure cell proliferation. Wound healing assay and Transwell assay were employed to evaluate cell migration and invasion, respectively. Cell apoptosis was determined by flow cytometry analysis, and protein level was measured by Western blotting. The experimental results demonstrated that chelidonine treatment inhibited cell viability and cell proliferation but facilitated cell apoptosis of melanoma cells. Besides, chelidonine suppressed melanoma cancer cell migration and invasion by attenuating epithelial-mesenchymal transition process. Moreover, chelidonine inhibited the activation of TLR4/NF-κB and PI3K/AKT pathways by downregulation of the protein level of TLR4, phosphorylated p65, phosphorylated PI3K, and phosphorylated AKT in melanoma cells. Furthermore, TAK-242 or LY294002 further enhanced the inhibitory effects chelidonine of on malignant cell behavior. In conclusion, our findings demonstrate that chelidonine effectively suppresses the malignancy of melanoma cells through the inhibition of TLR4/NF-κB and PI3K/AKT signaling pathways, suggesting its potential as a promising therapeutic agent for melanoma treatment.

INTRODUCTION

Melanoma, a malignant tumor originating from melanocytes, accounts for approximately 3% of all tumors [1]. Cutaneous malignant melanoma ranks third among cutaneous malignancies, comprising about 6.8% to 20% of cases [2,3]. Malignant melanomas can arise from congenital or acquired benign melanocyte nevi or undergo malignant transformation from dysplastic nevi [4]. In recent years, there has been a progressive increase in both the incidence and mortality rates of malignant melanoma, with individuals succumbing at an earlier age compared to other solid tumors [2,5]. The prognosis of melanoma patients remains unfavorable due to the absence of specific treatments beyond early surgical resection [6]. Consequently, timely diagnosis and treatment play pivotal roles in managing malignant melanoma.

Chelidonium majus L., commonly known as the greater celandine (Papaveraceae), has been found to possess significant antitumor and antiviral activities [7]. Chelidonine has been extensively studied for their therapeutic potential in western phytotherapy and traditional Chinese medicine in recent centuries [8,9]. Not only crude extracts of *C. majus L.* but also purified chelidonine possessed a broad range of biological properties including immunomodulatory, anti-inflammatory, antimicrobial, analgesic, and antiviral effects that make them promising candidates for further

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research into novel drug development strategies with potential clinical applications in various diseases [10-12]. Importantly, chelidonine has been extensively reported to play an anti-tumor role in several malignancies including pancreatic cancer and gastric carcinoma [13,14]. To be specific, chelidonine promoted cell apoptosis *via* targeting the GADD45A-p53 pathway in pancreatic cancer [13]. Besides, chelidonine contributes to mitotic slippage and induces apoptotic-like death of SGC-7901 cells in human gastric carcinoma [14]. Importantly, chelidonine facilitated cell apoptosis and inhibited the activation of STAT3 pathway in melanoma [15,16]. Nevertheless, the role of chelidonine in melanoma required further exploration.

The PI3K (phosphatidylinositol 3-kinase)-AKT pathway stands as a pivotal signaling network in cancer, with mounting evidence highlighting its crucial involvement in melanoma, often accompanied by concurrent activation of RAS-RAFMEK-ERK signaling [17,18]. Moreover, functional experiments have underscored the significant roles played by the PI3K-AKT pathway in both melanoma initiation and therapeutic resistance [19]. The rational advancement of such therapies will be expedited through strategies that harness the expanding comprehension of the intricate regulation and multifaceted functions exhibited by this pathway [19,20]. However, the relationship between chelidonine and PI3K-AKT pathway remains unclear in melanoma.

TLR4/NF-κB signaling is another important pathway in melanoma [21,22]. In detail, nuciferine attenuated cell growth in skin cutaneous melanoma by suppressing TLR4/NF-κB pathway [21]. Moreover, andrographolide repressed tumor growth by inhibiting the inactivation the TLR4/NF-κB signaling in melanoma [22]. Nevertheless, the relationship between chelidonine and TLR4/NF-κB pathway remains unclear in melanoma.

Based on published studies, we hypothesized that chelidonine inhibited melanoma cell malignancy by inactivating TLR4/NF-κB and PI3K/AKT pathways. Our study offers a promising therapeutic approach for the management of patients with melanoma.

METHODS

Cells and treatment

The human UM cell lines MEL270 and C918 were obtained from the Cell Bank of the Chinese Academy of Sciences and cultured in Dulbecco's Modified Eagle Medium (Gibco) containing 10% fetal bovine serum (FBS) (Gibco), 100 U/ml penicillin and 100 μg/ml streptomycin with 5% CO_2 at 37°C. Chelidonine (purity > 98%), TAK-242 (a TLR4 inhibitor) and LY294002 (a PI3K inhibitor) were purchased from MedChemExpress. Cells were treated with chelidonine (0.1, 0.2, 0.5, 1, 2, and 5 μM), TAK-242 (1 μM) and LY294002 (50 μM).

Cell counting kit-8 (CCK-8) assay

After the treatment of chelidonine, TAK-242 or LY294002, MEL270 and C918 cells were plated into 96-well plates (1×10^4 cells/ml) and incubated for 24 or 48 h. Then, CCK-8 solution (10 μl) was added and incubated for 2 h. The optical density was detected at 450 nm wavelength with a microplate reader.

Colony formation assay

After the treatment of chelidonine, TAK-242 or LY294002, MEL270 and C918 cells were seeded in 6-well plates (1,000 cells/well) for 2 weeks. After washing with cold phosphate buffered saline (PBS) (Gibco), the colonies in each well were fixed by ethanol and stained with crystal violet. The images were recorded under a computer-assisted microscope, the number of colonies were counted.

Flow cytometry analysis

MEL270 and C918 cells were plated in 6-well plates (3×10^5 cells/well) and treated with chelidonine, TAK-242 or LY294002 for 24 h. After detaching with 0.05% trypsin, washing with cold PBS, and centrifuging (1,500 rpm) for 5 min at 4°C, cells were gently suspended in the binding buffer and stained with Annexin V-FITC (5 μl, 10 μg/ml) in the dark for 15 min. Subsequently, cells were stained with propidium iodide (PI) (10 μl, 20 μg/ml) for another 5 min. At last, the apoptotic cells were analyzed by flow cytometry (BD FACSCanto II) immediately.

Wound healing assay

MEL270 and C918 cells were digested with trypsin and cultured in 6-well culture plate (2×10^5 cells/well) and treated with chelidonine, TAK-242 or LY294002. The linear scratches were made on the surface of cell monolayer by using a sterile 20-μl pipette tip perpendicular when the cell confluence reached about 100%. After washed with PBS, scratches-induced cell debris was removed gently. Hereafter, cells were cultured in serum-free me-

Table 1. Primary antibodies information

Protein	Dilution	Number	Origination
p-PI3K	1:1,000	ab182651	Abcam
PI3K	1:1,000	ab140307	Abcam
p-AKT	1:1,000	ab81283	Abcam
AKT	1:1,000	ab8805	Abcam
TLR4	1:1,000	ab22048	Abcam
p-p65	1:1,000	ab131100	Abcam
p65	1:1,000	ab32536	Abcam
E-cadherin	1:1,000	ab40772	Abcam
N-cadherin	1:1,000	ab76011	Abcam
GAPDH	1:1,000	ab8245	Abcam

dium. 24 h later, the scratch width was photographed in a microscope at 0 h and 24 h, and the wound closure rate was quantified by ImageJ software.

Transwell assay

The Transwell assay was operated with a Transwell chamber (8 μm; BD Biosciences) precoated with Matrigel (BD Biosciences). In brief, MEL270 and C918 cells were treated with chelidonine, TAK-242 or LY294002, and then placed into the upper chamber (serum-free), and the lower chamber was added with medium containing 10% FBS. Following the incubation for 24 h, cells in the low chamber were fixed with 4% formaldehyde and stained with 0.5% crystal violet. The invaded cells were observed and photographed with an inverted light microscope (Olympus Corporation).

Western blot

Total protein was extracted from MEL270 and C918 cells using RIPA lysis buffer (Beyotime). Subsequently, equal amounts of protein were loaded onto a 10% SDS-PAGE gel, and then subjected to electrophoresis at 120 V for 1.5 h. Subsequently, proteins were transferred onto polyvinylidene difluoride membranes (Bio-Rad), and blocked with 5% non-fat milk and incubated with the primary antibodies at 4°C overnight. Also, the membranes were incubated with the secondary antibody at room temperature for 1 h. At last, protein bands were visualized by the Chemiluminescent ECL assay kit (Amersham Life Sciences). The gray value was analyzed by Image J software. The detailed information of spe-

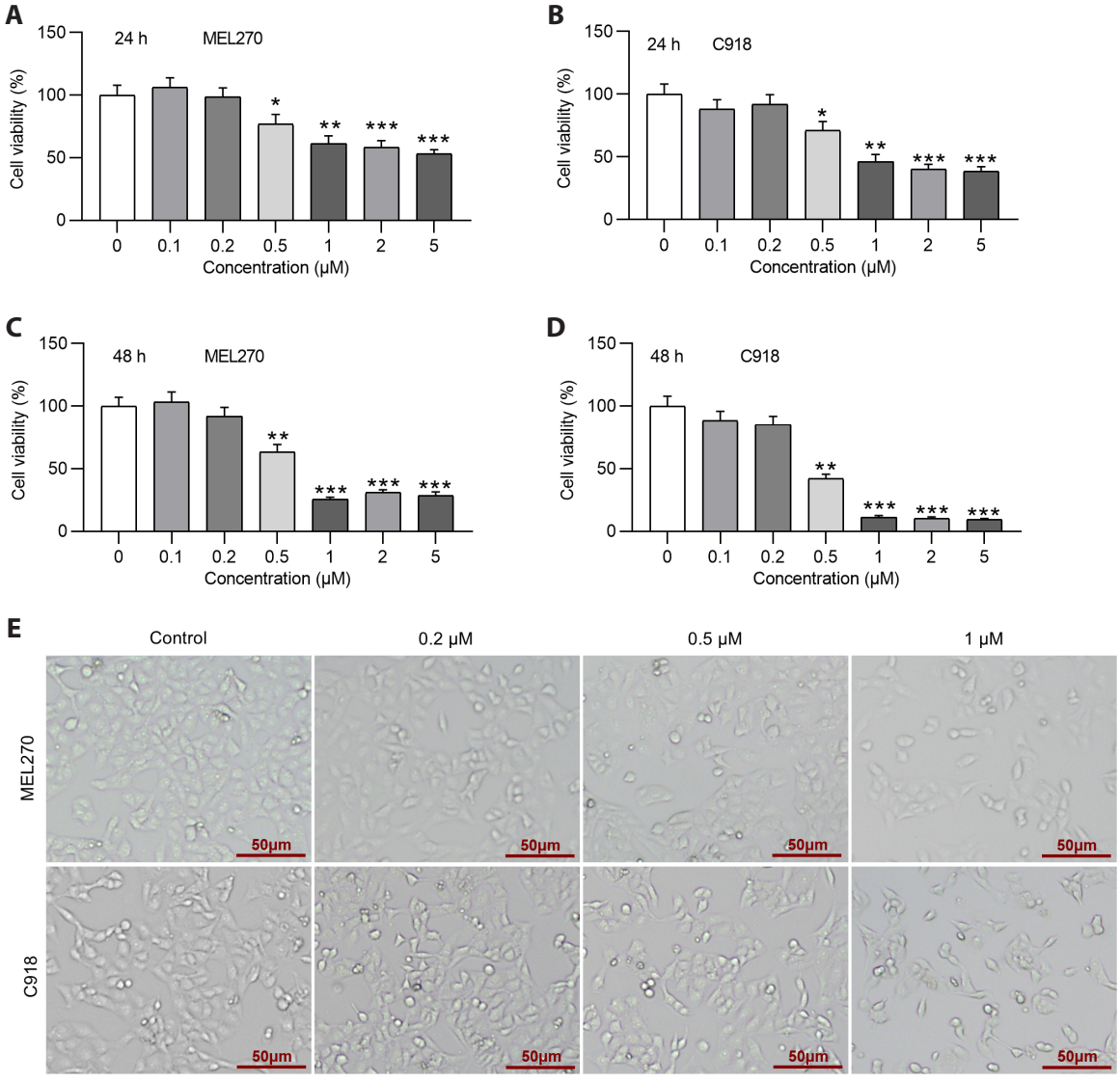

Fig. 1. The effect of chelidonine on cell viability of melanoma cells. (A-D) The MEL270 and C918 cell viability was evaluated by CCK-8 assay under chelidonine (0.1, 0.2, 0.5, 1, 2, and 5 μM) treatment for 24 or 48 h. (E) The representative images of cell morphology of MEL270 and C918 cells. Values are presented as mean ± SD. CCK-8, cell counting kit-8. *p < 0.05, **p < 0.01, ***p < 0.001.

cific primary antibodies was provided in Table 1.

Statistical analysis

The experimental data are shown as the mean ± standard deviation. Graphpad 7.0 software was used for statistical analysis. Differences between 2 groups were analyzed by Student's t-test, and differences among multiple groups were used one-way ANOVA followed by Tukey's *post-hoc* test. $p < 0.05$ was considered statistically significant.

RESULTS

The effect of chelidonine on cell viability of melanoma cells

To evaluate the inhibitory effects of chelidonine in melanoma cells, the MEL270 and C918 cells were treated with chelidonine (0.1, 0.2, 0.5, 1, 2, and 5 μM) for 24 or 48 h. The CCK-8 assay suggested that chelidonine (0.5, 1, 2, and 5 μM) treatment significantly inhibited cell viability of MEL270 and C918 cells in a dose-dependent manner (Fig. 1A-D). The low dose of chelidonine (0.1 or 0.2 μM) does not affect cell viability of MEL270 and C918 cells. In addition, the morphology of MEL270 and C918 cells was observed under a light microscope. Compared with control group, shrunken cell volume and reduced cell number were observed after chelidonine (0.5 or 1 μM) treatment (Fig. 1E). Hence, chelidonine treatment (0.5 or 1 μM) for 24 h is chosen for following assay. Taken together, chelidonine treatment inhibited cell viability of melanoma cells.

The effect of chelidonine on cell proliferation and apoptosis of melanoma cells

According to the colony formation assay, the number of colonies formed by MEL270 and C918 cells was markedly reduced by chelidonine treatment (Fig. 2A, B). On the contrary, the apoptosis rate of MEL270 and C918 cells is prominently enhanced by chelidonine treatment (Fig. 2C, D). To conclude, chelidonine treat-

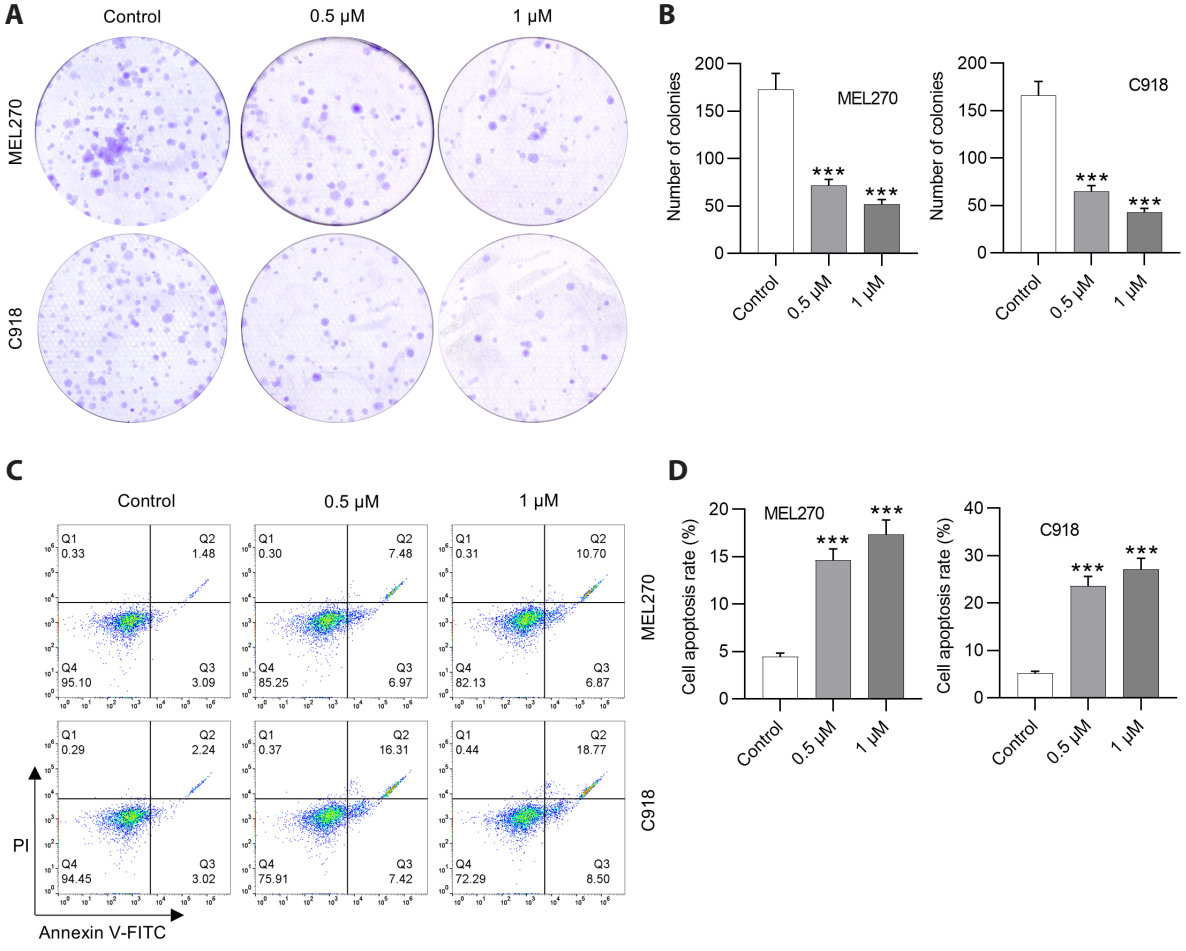

Fig. 2. The effect of chelidonine on cell proliferation and apoptosis of melanoma cells. (A, B) The representative images and quantification of colonies formed by MEL270 and C918 cells treated with chelidonine (0.5 or 1 μM). (C, D) The apoptotic MEL270 and C918 cells treated with chelidonine (0.5 or 1 μM) were determined by flow cytometry analysis. Values are presented as mean ± SD. PI, propidium iodide. ***$p < 0.001$.

ment repressed cell proliferation and facilitated cell apoptosis of melanoma cells.

The effect of chelidonine on cell migration and invasion of melanoma cells

Next, we explored the role of chelidonine in cell migration and invasion. The wound healing assay demonstrated that the wound closure rate of MEL270 and C918 cells is significantly reduced by chelidonine treatment (Fig. 3A, B). Moreover, transwell assay uncovered that the number of invaded MEL270 and C918 cells is also decreased by chelidonine treatment (Fig. 3C, D). Furthermore, chelidonine treatment not only increased E-cadherin protein but also decreased N-cadherin protein in MEL270 and C918 cells. All these results indicated that chelidonine suppressed laryngeal cancer cell migration and invasion of melanoma cells.

The effect of chelidonine on TLR4/NF-κB and PI3K/AKT signaling pathways in melanoma cells

We then explored the downstream pathway of chelidonine in melanoma cells. Western blotting analysis suggested that the protein level of TLR4 and phosphorylated p65 is downregulated by chelidonine treatment in MEL270 and C918 cells (Fig. 4A-C). Furthermore, the phosphorylated PI3K and AKT protein levels were also decreased by chelidonine treatment in MEL270 and

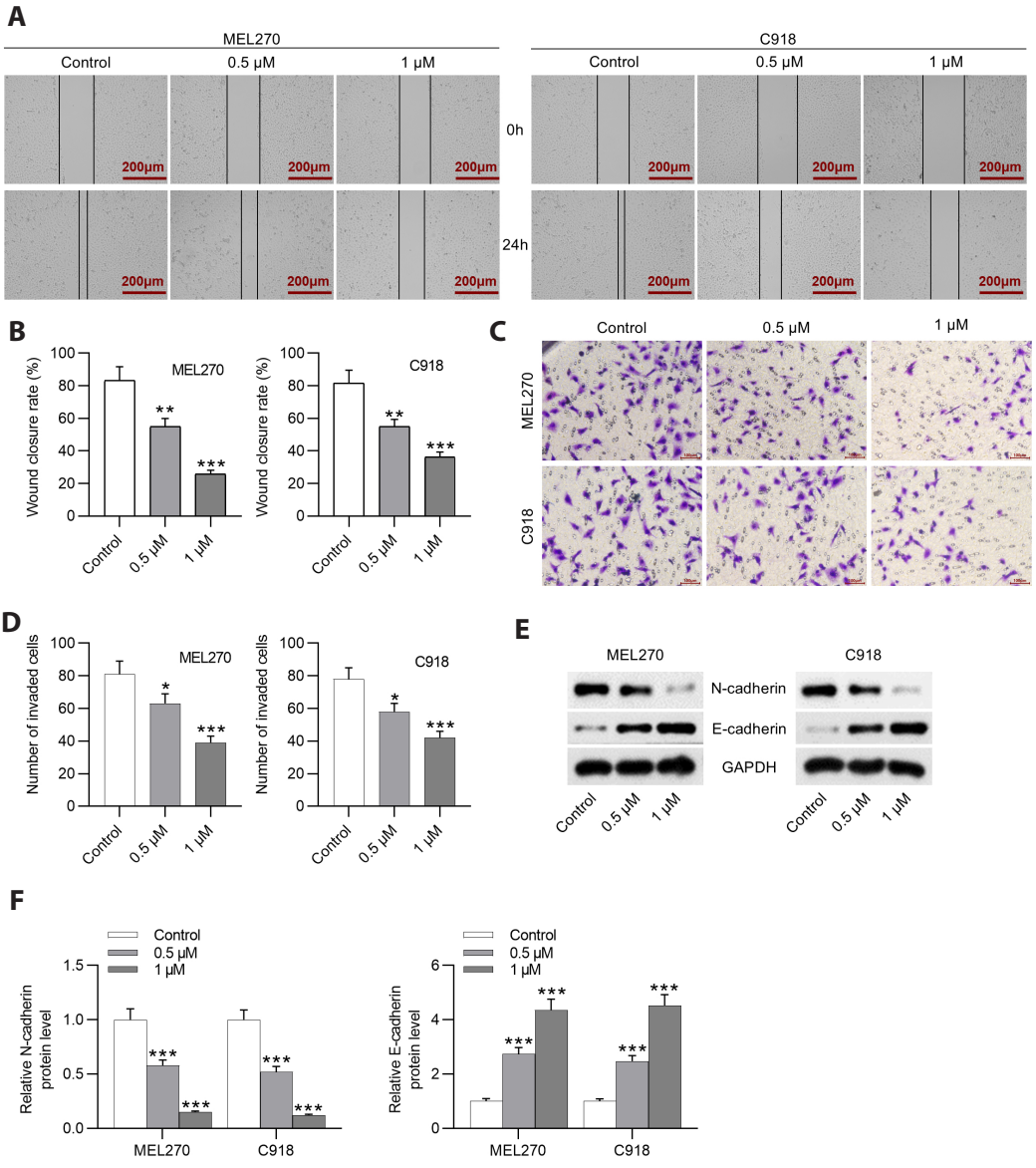

Fig. 3. The effect of chelidonine on cell migration and invasion of melanoma cells. (A–D) Cell migration and invasion of MEL270 and C918 cells treated with chelidonine (0.5 or 1 μM) were detected by wound healing and Transwell assays. (E, F) The E-cadherin and N-cadherin protein levels were analyzed by Western blot analysis in MEL270 and C918 cells treated with chelidonine (0.5 or 1 μM). Values are presented as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001.

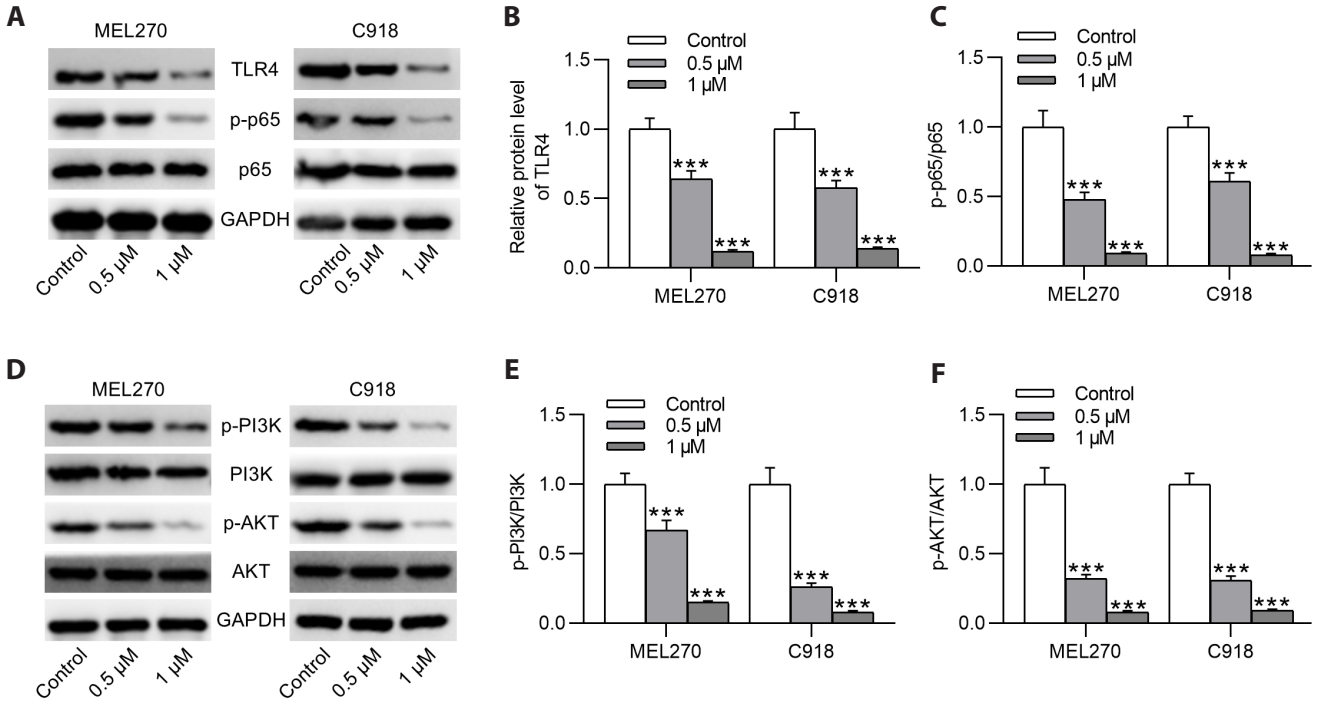

Fig. 4. The effect of chelidonine on TLR4/NF-κB and PI3K/AKT signaling pathways in melanoma cells. (A-F) The TLR4, phosphorylated and unphosphorylated p65, PI3K, and AKT protein levels were analyzed by Western blot analysis in MEL270 and C918 cells treated with chelidonine (0.5 or 1 μM). Values are presented as mean ± SD. ***p < 0.001.

C918 cells (Fig. 4D-F). To conclude, chelidonine inhibited the activation of TLR4/NF-κB and PI3K/AKT signaling pathways in melanoma cells.

LY294002 or TAK-242 enhances the inhibitory effects of chelidonine on cell malignancy of melanoma cells

To validate the effect of chelidonine on TLR4/NF-κB and PI3K/AKT pathways, TAK-242 or LY294002 were employed to treat melanoma cells. As shown in Fig. 5A, B, both chelidonine and the signaling inhibitors exhibited inhibitive effect onthese pathways, and the inhibitive effect of chelidonine on TLR4 and phosphorylated p65 proteins or phosphorylated PI3K and AKT proteins is strengthened by TAK-242 or LY294002 treatment, respectively. Moreover, chelidonine, TAK-242 or LY294002 treatment inhibited the cell viability, and chelidonine-mediated the decrease of cell viability is further reduced by TAK-242 or LY294002 treatment (Fig. 5C). Additionally, chelidonine, TAK-242 or LY294002 treatment attenuated colony formation of melanoma cells, and TAK-242 or LY294002 treatment also enhanced the inhibitive effect of chelidonine on colony formation (Fig. 5D). Besides, chelidonine, TAK-242 or LY294002 treatment promoted cell apoptosis of melanoma cells, and chelidonine-mediated the enhancement of cell apoptosis rate is increased by TAK-242 or LY294002 treatment (Fig. 5E, F). Moreover, chelidonine, TAK-242 or LY294002 treatment suppressed melanoma cell migration and invasion, and the suppressive effect of chelidonine on cell migration and invasion was also strengthened by TAK-242 or LY294002 treatment (Fig. 5G-J). Importantly, chelidonine, TAK-242 or LY294002 treatment upregulated E-cadherin protein and downregulated N-cadherin protein, and the inhibitive effect of chelidonine on epithelial-mesenchymal transition (EMT) process was strengthened by TAK-242 or LY294002 treatment (Fig. 5K). In summary, TAK-242 or LY294002 enhanced the inhibitory effects of chelidonine on malignant cell behavior.

DISCUSSION

Melanoma is an exceptionally malignant and aggressive tumor, characterized by a high incidence, extensive metastasis, radioresistant and chemoresistant [23]. The primary treatment modalities for melanoma encompass surgical resection, immunotherapy, and chemoradiotherapy [24]. Despite these advanced managements over the past decades, melanoma remains an insidious cancer type when diagnosed at advanced stages [23,25]. Therefore, the exploration of more efficacious and safer therapeutic agents for melanoma treatment assumes paramount importance as a pressing issue. In the current study, we further investigated the biological role and molecular mechanism of chelidonine. We revealed that chelidonine repressed cell proliferative, migrative and invasive abilities, but facilitated cell apoptosis of melanoma cells by inactivating TLR4/NF-κB and PI3K/AKT pathways.

Chelidonine, a critical component of *C. majus. L.*, was reported

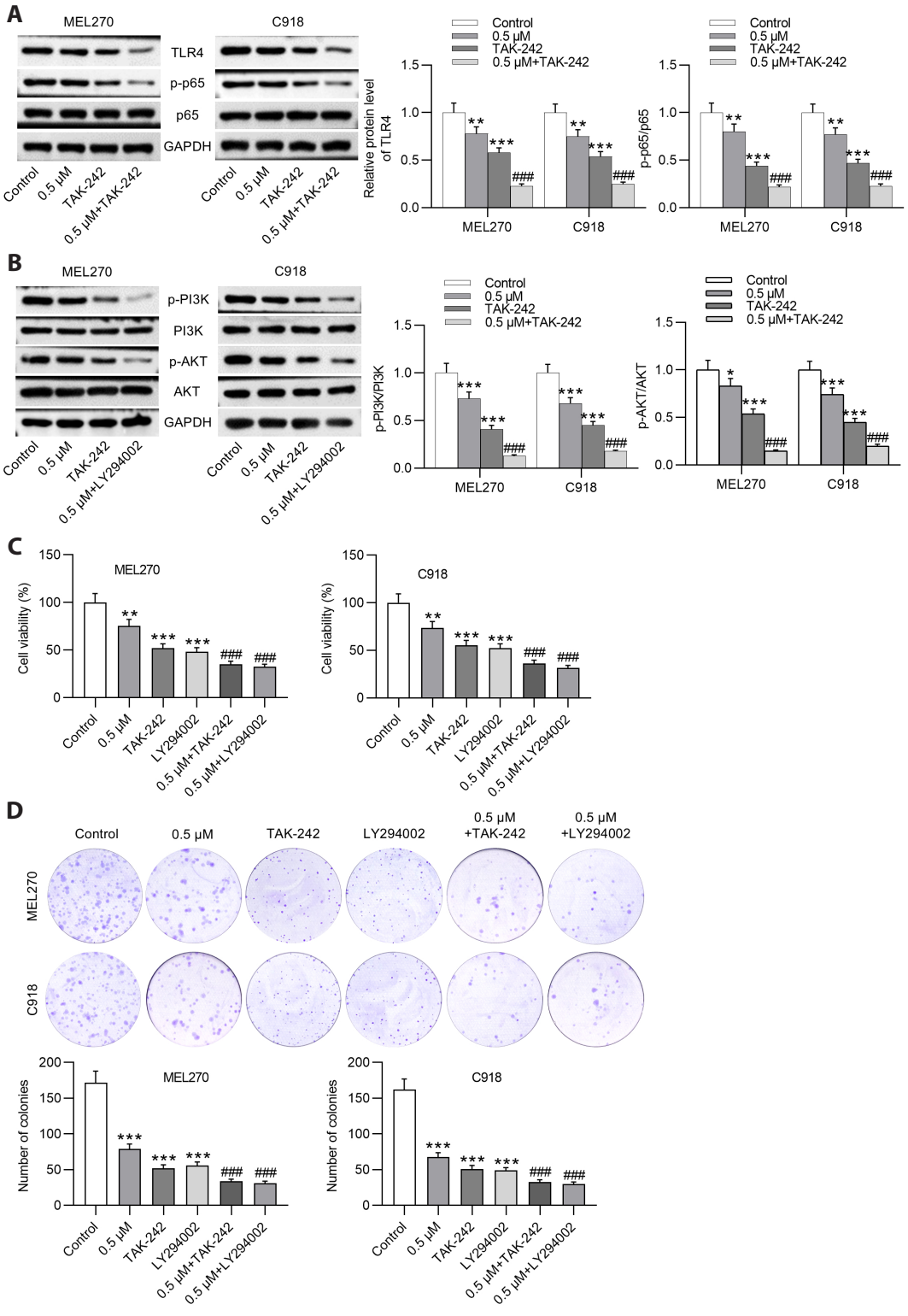

Fig. 5. LY294002 or TAK-242 enhances the inhibitory effects of chelidonine on cell malignancy of melanoma cells. (A, B) The protein level of TLR4, p65, PI3K, AKT, p-p65, p-PI3K, and p-AKT in MEL270 and C918 cells treated with chelidonine (0.5 μM) and LY294002 (50 μM) or TAK-242 (1 μM) was detected by Western blot. (C) Cell viability of MEL270 and C918 cells treated with chelidonine (0.5 μM) and/or LY294002 (50 μM) or TAK-242 (1 μM) was determined by CCK-8 assay. (D) The measurement of colony formation of MEL270 and C918 cells treated with chelidonine (0.5 μM) and/or LY294002 (50 μM) or TAK-242 (1 μM). (E, F) The apoptotic MEL270 and C918 cells treated with chelidonine (0.5 μM) and LY294002 (50 μM) or TAK-242 (1 μM) were determined by flow cytometry analysis. (G-J) Cell migration and invasion of MEL270 and C918 cells treated with chelidonine (0.5 μM) and/or LY294002 (50 μM) or TAK-242 (1 μM) were tested by wound healing assay and Transwell assay, respectively. (K) The measurement of E-cadherin and N-cadherin protein levels in MEL270 and C918 cells treated with chelidonine (0.5 μM) and/or LY294002 (50 μM) or TAK-242 (1 μM). Values are presented as mean ± SD. CCK-8, cell counting kit-8; PI, propidium iodide. *p < 0.05, **p < 0.01, ***p < 0.001 *vs*. control group. ###p < 0.001 *vs*. 0.5 μM group.

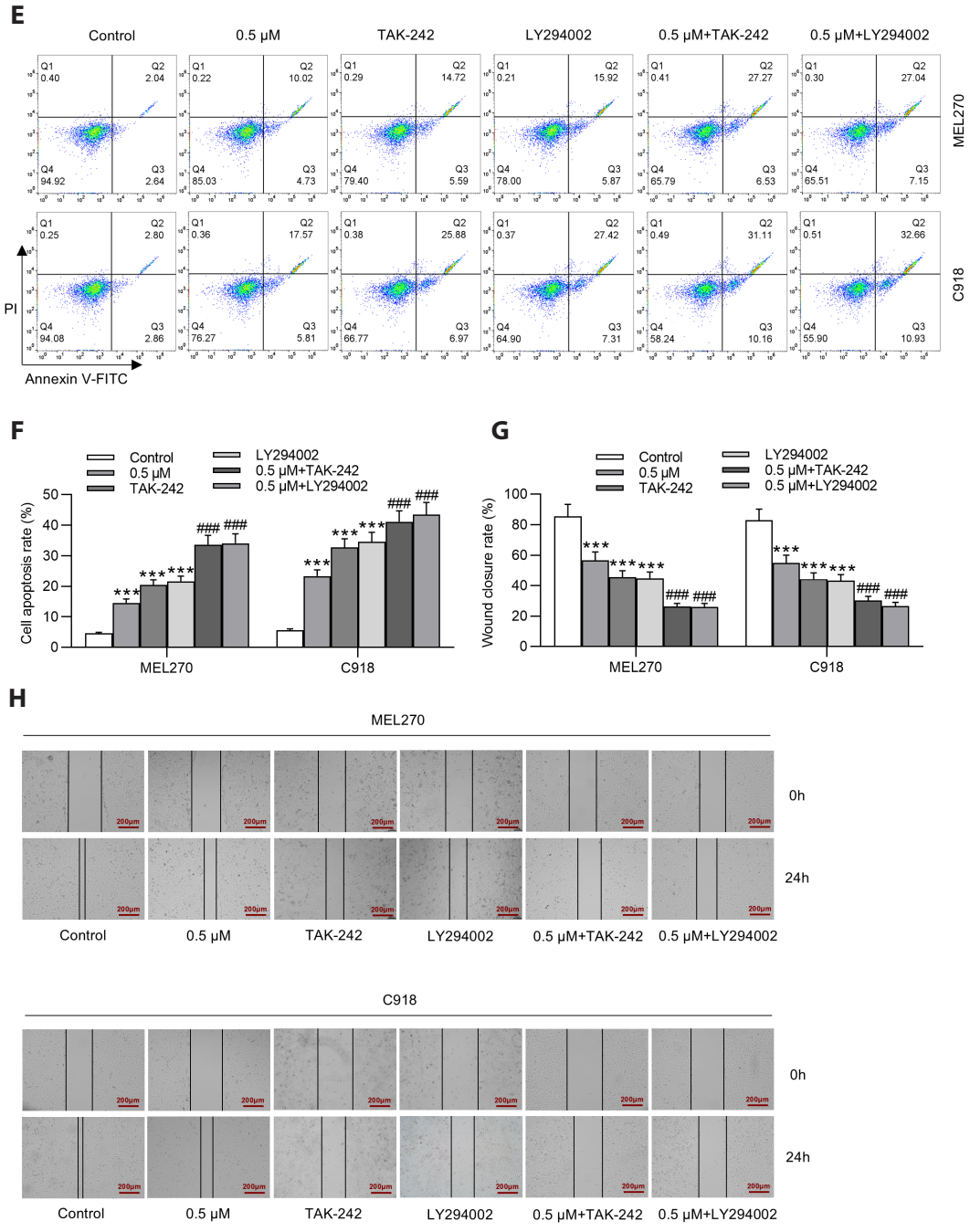

Fig. 5. Continued.

to possess various biological and pharmacological activities including ani-inflammation, antivirus and anti-cancer [26-28]. Notably, chelidonine was identified to effectively suppress tumor cell growth and enhance tumor cell apoptosis. In detail, chelidonine inhibited gefitinib-resistant cell proliferation *via* targeting EGFR-AMPK pathway in non-small cell lung cancer [29]. Besides, chelidonine contributed to apoptotic-like death of gastric carcinoma cells by activation of caspase-dependent pathway [14]. Similarly, chelidonine also attenuated cell growth but promoted cell apoptosis in melanoma. Cell migration and invasion were critical steps of melanoma metastasis, which induced high recurrence and poor prognosis [30,31]. Coincidentally, chelidonine was proposed to repressed cell migration and invasion in head and neck cancer and breast cancer [32,33]. In our study, chelidonine exerted anti-migratory and anti-invasive effects on melanoma cells. Importantly, chelidonine attenuated EMT process by upregulating E-cadherin and downregulating N-cadherin. Although chelidonine was reported to facilitate the apoptosis of NRAS-mutant cancer cells, B16F10 cells and OCM-1 cells [34-36], our study further focused on the effect of combination chelidonine and inhibitors

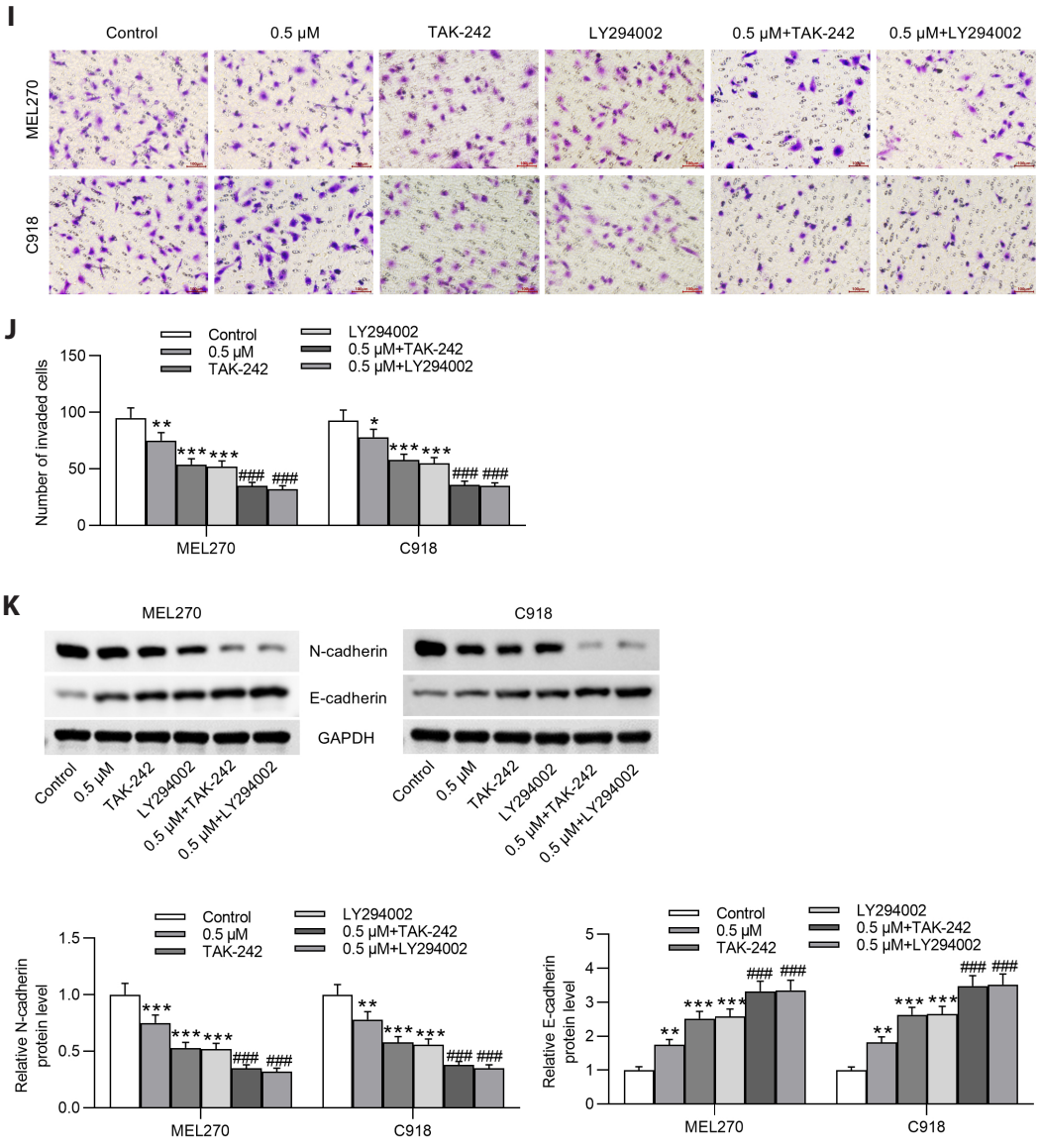

Fig. 5. Continued.

of these pathways on melanoma cell phenotype. The combination use of chelidonine and TAK242 or LY294002 further strengthened the inhibitive effect of chelidonine on malignant phenotype of melanoma cells.

In mechanism, several downstream signaling pathways were proven to be regulated by chelidonine. To be specific, chelidonine activated caspase-dependent pathway in gastric cancer cells and p53 pathway in pancreatic cancer cells but inactivated NF-κB pathway in HCT116 cells and PI3K/AKT pathway in Hela cells [9,10,13,14,37]. Importantly, NF-κB and PI3K/AKT pathways were widely reported to contribute to cell malignancy in melanoma. Hence, we anticipated that chelidonine potentially targeting NF-κB and PI3K/AKT pathways in melanoma cells. As expected, chelidonine inhibited the activation of NF-κB and PI3K/AKT pathways by downregulation of the protein level of TLR4, phosphorylated p65, phosphorylated PI3K, and phosphorylated AKT. Moreover, chelidonine exerted similar effects with TAK-242 (TLR4 inhibitor) or LY294002 (PI3K inhibitor) on the phenotype of melanoma cells. Moreover, the combination use of chelidonine and TAK-242 (or LY294002) further enhanced the inhibitive effect of chelidonine on TLR4/NF-κB and PI3K/AKT pathways.

In summary, we discovered that chelidonine attenuated melanoma cell malignancy by inactivating TLR4/NF-κB and PI3K/AKT pathways. We further evaluated the effect of combination use of chelidonine and TAK-242 (or LY294002) on melanoma cell phenotype for the first time, which offered the experimental and theoretical foundation for the clinical trials of chelidonine in the treatment of melanoma. Nevertheless, the role of chelidonine *in vivo* remains to be explored in the future.

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None to declare.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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