Luteolin induced G2 phase cell cycle arrest and apoptosis on non-small cell lung cancer cells

Xueting Cai a,b, Tingmei Ye c, Chao Liu b, Wuguang Lu b, Min Lu a, Juan Zhang d, Min Wang a,*, Peng Cao b,*

a School of Life Science and Technology, China Pharmaceutical University, Nanjing 210009, Jiangsu, China
b Laboratory of Cellular and Molecular Biology, Jiangsu Province Institute of Traditional Chinese Medicine, Nanjing 210028, Jiangsu, China
c Department of Biology, School of Chemistry and Life Sciences, Lishui University, Lishui 323000, Zhejiang, China

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A B S T R A C T
In this study, we investigated the underlying molecular mechanism for the potent cell cycle inhibition and pro-apoptotic effect of luteolin (2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4-chromenone) on human non-small-cell lung carcinoma cell line A549. MTT assay showed that luteolin had obvious cytotoxicity on A549 with IC50 of 40.2 μM at 48 h. Pro-apoptotic effect of luteolin on A549 cells was demonstrated by Hoechst 33258 staining assay and annexin V-FITC/PI double staining analysis. A great quantity of apoptotic cells and increasing G2 phase cells were observed by flow cytometry. Western blotting assay revealed that luteolin activated JNK, increased Bax, promoted procaspase-9 cleavage and activated caspase-3 at last. Assay using TNFα, an active agent of NF-κB, showed that pretreatment of A549 cells with luteolin could inhibit TNFα-induced trans-nuclear of NF-κB. In summary, luteolin displayed a significant cytotoxic effect through cell cycle arrest and apoptosis induction in A549 cells. Pro-apoptotic effect was implemented via activating JNK and inhibiting translocation of NF-κB (p65). These results suggested that luteolin might have therapeutic potential against NSCLC.

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1. Introduction

Lung cancer is the most common cause of cancer-related death. Non-small-cell lung cancer (NSCLC) accounting for 80–85% of all cases with approximately 70% of patients presenting with advanced disease (stage IIB with pleural effusion or stage IV) at the time of diagnosis (Stewart, 2010). Current first-line treatments for patients with NSCLC include chemotherapy with a platinum agent in combination with a taxane or other cytotoxic agent such as gemcitabine or vinorelbine. Some patients in the late period of NSCLC respond to chemotherapy initially, but gradually they become resistant. Thus, new methods to improve the clinical response to chemotherapy are required.

The significance role of vegetables and fruits in prevention and treatment of cancer is due to their high polyphenol content, particularly of flavonoids (Ramos, 2007). Flavonoids are well known for its effects on xenobiotic and carcinogen metabolism (Moon et al., 2010; Zhang et al., 2008), but the mechanism of luteolin affected cancer cells was not addressed. Therefore, we used the non-small-cell lung carcinoma cell line A549 as a model to examine the therapeutic effects of luteolin.

2. Materials and methods

2.1. Materials

Luteolin (Fig. 1) was purchased from Nanjing TCM Institute of Chinese Materia Medica, China. Luteolin was dissolved in dimethyl sulfoxide (DMSO) and was used in all experiments. RPMI-1640 medium, heat-inactivated fetal bovine serum (FBS), penicillin and streptomycin were purchased from Gibco, USA. Caspase-3, p-MEKK1, MEKK1, goat anti-mouse IgG-HRP and goat anti-rabbit IgG-HRP, Goat anti-rabbit IgG-FITC antibodies were obtained from Santa Cruz, USA. Mouse anti-Bax was obtained from BD Biosciences, USA. Bcl-xL, oil, and tea (Sasaki et al., 2003). In cellular studies, luteolin has been found to possess anti-oxidant, anti-inflammatory/anti-allergic, anti-tumorigenic, and radical action (Ashokkumar and Sudhandiran, 2008; Brown et al., 1998; Galati et al., 2001). Luteolin was reported to inhibit the development of a series of solid tumors (colon HT-29, HCT116, hepatic HepC2, SK-Hep-1, PLC/PRF/5, Hep3, cervical Hela, oral SCC-4) (Chang et al., 2005; Lee et al., 2005, 2006; Lim do et al., 2007; Plaumann et al., 1996; Schutte et al., 2008; Xavier et al., 2009; Yang et al., 2008; Yee et al., 2003; Zhang et al., 2008), but the mechanism of luteolin affected on human lung cancer cells was not addressed. Therefore, we used the non-small-cell lung carcinoma cell line A549 as a model to examine the therapeutic effects of luteolin.

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PARP, Caspase-8, Cyclin A, p-I

Molecular structure of luteolin (C15H10O6, CAS No: 491-70-3, Mol. Wt.: 286.24).

Fig. 1. Molecular structure of luteolin (C15H10O6, CAS No: 491-70-3, Mol. Wt.: 286.24).

2.5. Cell cycle analysis

The NSCLC cell line A549 was purchased from the American Type Culture Collection. Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. Cultures were maintained in a humidified environment with 5% CO2 at 37°C.

2.6. Hoechst 33258 staining

Hoechst 33258 staining was used to visualize nuclear change and apoptotic body formation. At the end of luteolin treatment, attached cells were washed twice with PBS and fixed with 4% formaldehyde at 4°C for 30 min. The fixing solution was removed and cells were washed twice with PBS before staining with Hoechst 33258 (KeyGEN, Nanjing, China). After staining for 10 min, cells were washed again and observed under a fluorescence microscope (Zeiss Axio Observer A1) at 340 nm.

2.7. Annexin-V/PI double staining assay

A549 cells were treated with 40 μM luteolin for 12 h or 24 h. Then they were harvested, washed and resuspended with PBS. Apoptotic cells were determined with an FITC Annexin V Apoptosis Detection Kit (BD Biosciences, USA) according to the manufacturer's protocol. Briefly, the cells were washed and subsequently incubated for 15 min at room temperature in the dark in 100 μl of 1x binding buffer containing 5 μl of Annexin V-FITC and 5 μl of PI. Afterward, apoptosis was analyzed by fluorescence microscope and FACScan laser flow cytometer.

2.8. Western blotting analysis

A549 cells were cultured in RPMI 1640 until mid-log phase and then incubated with 20, 40, 60 or 80 μM luteolin for 24 h. Proteins were isolated by lysis buffer (Beyotime, China) and measured using the Nanodrop 1000 Spectrophotometer (Thermo, USA). Protein samples were separated on 13% SDS–polyacrylamide gels (SDS–PAGE) and transferred onto the PVDF membranes (Millipore, USA). After blocked with 1% BSA in TBST for 2 h, membranes were incubated with primary antibodies overnight at 4°C. Blots were washed and incubated with secondary antibodies for 1 h at room temperature. Membranes were again washed three times with TBST and developed using enhanced chemiluminescence (Luminata Crescendo Western HRP substrate, Millipore, USA). Membranes were then exposed to films.

2.9. NF-κB (p65) translocation

The effect of luteolin on TNFα induced nuclear translocation of p65 was examined using an immunocytochemical method. A549 cells were plated in 24 well plates at a density of 5 × 104 cells per well. The next day, medium was changed to serum-free medium for 12 h. Cells were pretreated with 40 μM luteolin for 6 h before stimulation with 20 ng/ml TNFα (PeproTech, USA) for 10 min. Treated cells were terminated by washing with ice cold PBS followed by fixation with 4% formaldehyde for 30 min at 4°C. Cells were permeabilized with 0.05% NP-40 (Amresco, USA) for 30 min at room temperature, washed with PBS and blocked with 1% bovine serum albumin (BSA) for 1 h. Rabbit anti-p65 antibody was added and incubated overnight at 4°C. After washing with PBS for three times, goat anti-rabbit IgG-FITC was added and incubated for 1 h at room temperature. Fluorescence cells were observed and photographed under a fluorescence microscope. The nuclear proteins after treating with luteolin or/and TNFα were extracted using Nuclear and Cytoplasmic Extraction Reagents (Pierce, USA) followed the instruction. The extracts were then resolved on 13% SDS–PAGE and analyzed by western blotting using antibodies against p-p65.
2.10. Statistical analysis

Values were expressed as means ± standard deviations. Statistical analysis was performed by one-way analysis of variance. When significance was detected, the t-test for multiple comparisons was performed on the data from experimental groups. A probability value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Cytotoxicity effect of luteolin on A549 cells

Differences in cell morphology were observed between luteolin-treated and control cells by light microscopy. The most conspicuous change observed in luteolin-treated cells included cell shrinkage and extensive detachment of the cells from the cell culture substratum (Fig. 2A). These changes, which were characteristic of cell apoptotic death, became visible after 12 h treatment, but were absent in control cells. The morphological change became more remarkable with increased time of drug treatment (images showed here were photographed after 24 h treatment, other time points were not shown).

The inhibition effect of luteolin on cell growth was assessed by the commonly used MTT assay at different intervals (12, 24, 48 and 72 h) of treatment. Luteolin treatment significantly inhibited the growth of A549 cells in both concentration- and time-dependent manner (Fig. 2B). The IC$_{50}$ values at 24, 48 and 72 h were 68.1, 45.2 and 35.2 μM, respectively.

3.2. Luteolin treatment caused G2 phase cell cycle arrest with inhibition of the expression of cyclin A and phosphorylation of CDC2, Rb

To test whether luteolin could affect the cell cycle of A549 cells, cells treated with DMSO or different concentration of luteolin for 24 h were subjected to flow cytometric analysis after DNA staining. As shown in Fig. 3A and B exposure of A549 cells to growth suppressive concentrations of luteolin resulted in a statistically significant increase in the G2 phase that was accompanied by a decrease in the G1 phase. For example, the percentage of G2 phase increased by ~1.97-fold on treatment of A549 cells with 20 μM luteolin compared with control. Further research (Western bolt assay) showed that the expression of G2 phase cell cycle related proteins (cyclin A, p-CDC2 and p-Rb) were significantly decreased in luteolin-treated A549 cells (Fig. 3C). GAPDH was used as reference.

3.3. Pro-apoptotic effect of luteolin on A549 cells

The occurrence of apoptosis was further verified by Hoechst staining, which detects chromatin condensation, one of the hallmarks of apoptotic cell death. Differences were observed in the nuclei of luteolin-treated and -untreated A549 cells after staining with Hoechst 33258 (Fig. 4A). The Hoechst 33258 dye stained morphologically normal nuclei dimly blue, whereas luteolin-treated cells demonstrated smaller nuclei with brilliant blue staining (white arrows). The change in nuclear morphology was initially observed after 12 h of luteolin treatment and increased thereafter. These results demonstrated that luteolin induces morphological changes characteristic of cell apoptotic death. Additional evidence for the occurrence of apoptosis was obtained by double staining of the cultures with PI and annexin V-FITC. Annexin V was a protein that binds with high affinity to phosphatidylserine, which is translocated from the inner to the outer membrane leaflet early in the apoptotic process. As shown in Fig. 4B, control cells stained negative for both PI (red) and annexin V-FITC (green). Luteolin-induced cells, on the other hand, showed many annexin V-positive, PI-negative cells, indicating that they were at an early stage of apoptosis. The double positive staining of particular cells revealed that these cells were at a late apoptotic stage or necrotic. Take together, these findings provided strong evidence that luteolin pro-apoptosis on A549 cells.
3.4. Luteolin promote apoptosis through both NF-κB and JNK passway

The molecular mechanism for the potent pro-apoptotic effect of luteolin on A549 cells was further studied. Western blotting analysis was done as described in Section 2. As showed in Fig. 5A, luteolin increased phosphorylation of JNK1/2/3, which could induce apoptosis through promoting Bax translocation to mitochondria, then activated caspase-9. The increased expression of pro-apoptotic protein (Bax) and decreased expression of anti-apoptotic protein (Bcl-XL) were observed, with a concomitant increase in the levels of caspase-3 and cleaved poly-ADP-ribose polymerase (PARP) in A549 after treatment for 24 h. Luteolin activated caspase-9 but not caspase-8 indicted that luteolin induced apoptosis in A549 cells might through a mitochondria-dependent (intrinsic) apoptosis passway. We also found that the phosphorylation of NF-κB(p65) was decreased under the pressure of luteolin (Fig. 5B). Simultaneously, the phosphorylation level of the inhibitory protein, IκBα, which bounded to a NF-κB heterodimer (p50/p65) was down-regulated, so did the up-stream protein MEKK1. These results suggested that luteolin maybe aimed at MEKK1's phosphorylation, the decreased p-MEKK1 resulted in abate release of IκBα from IκBα/p50/p65 complex. p65 could not trans-nuclear caused apoptosis in A549 cells.

3.5. Luteolin inhibited TNFα induced NF-κB (p65) translocation

Western blotting assay showed that luteolin affect the IκBα/NF-κB signal passway. In order to demonstrate weather luteolin's pro-apoptotic effect in A549 cells part due to inhibit translocation of NF-κB, we used immunofluorescence technique to visualize the
dynamic movement of the p65 between the cytoplasm and nucleus. To manipulate the p65 movement, we used TNFα, a known NF-κB activator. The majority of the p65 subunit was detected in the cytoplasm of A549 cells in control and luteolin groups, but the addition of 20 ng/mL TNFα resulted in complete translocation of p65 into the nucleus and pretreatment with luteolin inhibited TNFα induced NF-κB (p65) translocation (Fig. 6A). The nuclear protein extracts analyzed by western blotting showed that luteolin completely suppressed the TNFα induced activation of p65 (Fig. 6B). Histon H3 was used as reference.

![Figure 4](image1.png)

**Fig. 4.** Luteolin-induced apoptosis of A549 cells. (A) nuclear staining of A549 cells with Hoechst 33258. Compared to control (DMSO), luteolin-treated cells demonstrated smaller nuclei with brilliant blue staining (white arrows); (B) Flow cytometric analysis of annexin V-FITC/PI double-staining. A549 cells were untreated or treated for 12 h and 24 h with 40 μM luteolin. Untreated cells were primarily Annexin V-FITC and PI negative, indicating that they were viable and not undergoing apoptosis. After treatment, there were primarily two populations of cells: Cells that were viable and not undergoing apoptosis (Annexin V-FITC and PI negative) and cells undergoing apoptosis (Annexin V-FITC positive and PI negative). A minor population of cells were observed to be Annexin V-FITC and PI positive, indicating that they were in end stage apoptosis or necrotic.

![Figure 5](image2.png)

**Fig. 5.** Western blotting analysis of IκBα/NF-κB and JNK passway related proteins. Total cell lysates of A549 cells treated with or without luteolin for the indicated time were analyzed by SDS-PAGE and, subsequently, immunoblotted with antibodies against p-JNK1/2/3, JNK1/2/3, Bax, Bcl-XL, caspase-3, caspase-8, caspase-9, PARP, MEKK1, p-MEKK1, MEKK1, p-IκBα, and GAPDH.
The JNK family belongs to the mitogen-activated protein kinase (MAPK) super family, which also includes the extracellular signal-regulated kinases (ERKs) and the p38 MAPK family. Although there are exceptions, a bulk of evidence suggests that the activation of ERK pathway increases the cell death threshold in an unknown manner (Ishikawa and Kitamura, 1999). Conversely, the activation of JNK and p38 kinase cascades is generally associated with an enhanced activation of the apoptotic program (Ichijo et al., 1997). Many researches have done with luteolin, revealed that luteolin could either activate JNK (Lee et al., 2005; Shi et al., 2004) or down regulate JNK phosphorylation (Ando et al., 2009; Jang et al., 2008; Kimata et al., 2000). Consistent with these reports, our results revealed that luteolin activated JNK in a concentration-dependent manner in A549 cells accompany an induction of apoptosis. Luteolin decreased the expression of Bax and increased the expression of Bcl-XL, with a concomitant increase in the levels of caspase-3 and cleaved PARP. To investigate the caspase cascade reaction through which apoptosis passway, we detected the expression of caspase-8 and caspase-9 in luteolin treatment A549 cells. The results showed that luteolin could active caspase-9 and promote it to cleave but not caspase-8. That suggested luteolin maybe induce apoptosis through a mitochondria-dependent (intrinsic) apoptosis passway.

Although the NF-κB signaling pathway has been implicated as one of the luteolin targets, an affect on translocation of NF-κB and an inhibitory effect of luteolin on the catalytic activity of MEKK1 has not been shown (Shi et al., 2004). The phosphorylation of MEKK1, an important kinase for the stimulation of IKKβ phosphorylation of IκBα was prominently inhibited by luteolin. Our research indicated luteolin may used as a new inhibitor agent of MEKK1, more verification experiments are in progress. TNFx is well known for its two aspects signaling pathways that determine whether a cell lives or dies. Whereas one pathway is conducive to cell death, the other leads to activation of NF-κB transcription factors and the inhibition of apoptosis (Nagata, 1997). Luteolin could inhibit TNFx induced NF-κB translocation, considering this, the combination of luteolin and TNFx could block TNFx induced anti-apoptotic effect. This effect suggests that luteolin may be more effective in combination with TNFα at relatively low concentration in treatment of A549 cells. Combination of Traditional Chinese Medicine with chemotherapeutic drugs or cytokines was a new cancer treatment options (Li et al., 2011). These results have revealed the potential efficacy of luteolin for NSCLC therapy, and have a foundation for further investigations.

Luteolin could inhibit SKOV3.ip1 tumor growth in a mouse xenograft model (Chiang et al., 2007). Tumor growth and angiogenesis in A431 tumor xenograft model was significantly inhibited by luteolin (Bagli et al., 2004). The average human daily intake of luteolin is approximately 16 mg per day (Hertog et al., 1993). Based on the above results, we assume that by increasing the daily dietary levels of luteolin may prevent NSCLC and be used to treat NSCLC patients, as well. In conclusion, our study indicates that...
luteolin inhibits cell growth, and induces G2 arrest and apoptotic cell death of A549 cells. Pro-apoptotic effect was implemented via activating JNK and inhibiting translocation of NF-κB (p65) (Fig. 7). Therefore, luteolin may act as a potential candidate for NSCLC chemoprevention and chemotherapy.

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