

Anti-proliferative and chemosensitizing effects of luteolin on human gastric cancer AGS cell line

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Abstract To investigate the anti-proliferative and chemosensitizing effects of luteolin on human gastric cancer, gastric cancer AGS cells were treated with luteolin and/or other chemotherapeutic agents. Cell growth was assessed by MTT assay, cell cycle and apoptosis were assessed by flow-cytometric analysis, and the expression of major proteins regulating cell cycle and apoptosis was also detected. The results showed that luteolin inhibited the growth of gastric cancer cells in a dose- and time-dependent manner. Flow cytometry revealed that the percentage of cells at G2/M phase increased dose-dependently. The protein levels of Cdc2, Cyclin B1 and Cdc25C were reduced and p21/cip1 was up-regulated after the treatment with luteolin. Furthermore, luteolin induced apoptosis in gastric cancer AGS cells. Western blotting showed that luteolin treatment significantly increased the levels of pro-apoptotic proteins, including Caspase-3, 6, 9, Bax, and p53, and decreased the levels of anti-apoptotic protein Bcl-2, thus shifting the Bax/Bcl ratio in favor of apoptosis. It was also demonstrated that a combinational treatment of cisplatin and luteolin induced more effectively cell growth inhibition, compared to cisplatin treatment alone. These findings indicate the anti-proliferative and chemosensitizing effects of

luteolin on human gastric cancer AGS cells and luteolin may be a promising candidate agent used in the treatment of gastric cancer.

Keywords Luteolin · Gastric cancer · Cell cycle arrest · Apoptosis · Chemosensitizing

Introduction

Gastric cancer continues to be a significant health issue, which is second only to lung cancer as a leading cause of cancer deaths worldwide [1–3]. The incidence of gastric cancer is reported to be especially high in Asia, South America, and Eastern Europe. Surgery remains the mainstay of any curative treatment; however, approximately two-thirds of patients diagnosed with gastric cancer have unresectable locally advanced and/or metastatic disease [4]. In this way, cytotoxic chemotherapy has been demonstrated to be effective. Nevertheless, patient prognosis remains very poor, and therapy-associated toxicity remains a problem [5]. Thus, it is necessary to find new compounds and optimized combinational treatment for gastric cancer.

Flavonoids, a kind of compounds that widely exist in the plant kingdom, exhibit a wide range of biological effects, such as anti-oxidant, anti-microbial, anti-inflammatory, anti-mutagenic, anti-clastogenic, and anti-carcinogenic [6–9]. Luteolin is one of the most common flavonoids and has been found to have anti-carcinogenic properties. Luteolin shows strong anti-proliferative activity against different human cancer cell lines, including lung cancer, myeloid leukemia, prostate cancer, and pancreatic cancer [10]. Furthermore, a recent study showed that luteolin significantly enhanced the cancer therapeutic activity of cisplatin via p53 stabilization and accumulation both

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in vivo and in vitro [11], which may provide a novel way for combinational chemotherapy of cancers. Apoptosis and cell cycle regulation were supposed to be mechanisms of the anti-cancer effects of luteolin. Some studies indicated that luteolin has diverse roles on various kinase signal transduction pathways regulating cell growth, cell cycle control, or apoptosis in multi-type cancer cells [12–14]. However, the role of luteolin in gastric cancer is still not well understood, because few studies have reported the effects of luteolin on the growth of gastric cancer cells. Therefore, we perform this study to examine whether and how luteolin regulates cell cycle progression and/or apoptosis in gastric cancer AGS cell line, and whether luteolin sensitizes the anti-cancer effect of common chemotherapeutic agents for human gastric cancer.

Materials and methods

Reagents and chemicals

RPMI1640 and other culture materials were purchased from Invitrogen Corporation (Gaithersburg, MD, USA). Antibodies for p21/Cip1, p27/kip1, Cdc2, Cyclin B1, Cdc25C, p53, caspase-8, and cytochrome C were purchased from CellSignal Inc. (Beverly, MA, USA), caspase-3, 6, 9, Bcl-xL, Bcl-2, and Bax were purchased from Chemicon (Temecula, CA, USA). Luteolin, cisplatin, antibody for β -actin, dimethyl sulfoxide (DMSO), propidium iodide, and other chemical agents were purchased from Sigma-Aldrich (St Louis, MO, USA).

Cell culture

Human gastric cancer cell line AGS was obtained from American Type Culture Collection. Cells were cultured in RPMI1640 medium supplemented with heat inactivated 10% fetal bovine serum made by Hyclone (Logan, UT, USA), 100 μ g/ml penicillin, 100 μ g/ml streptomycin, and 100 μ g/ml amphotericin B. The cells were grown in a humidified incubator at 37°C and a 5% CO₂ air atmosphere.

MTT cell viability assay

Cell viability was determined using the conversion of MTT to formazan via mitochondrial oxidation. Cells (5,000 cells/well) were treated with luteolin (0, 10, 20, 40, 80 μ M) for 24, 48, and 72 h. Then, MTT solution was added to each well at a final concentration of 1 mg/ml per well and the plates were incubated at 37°C for another 4 h. After incubation, 200 μ l DMSO was added to each well to dissolve the formazan and absorbance was read at 550 nm using a spectrophotometric

microplate reader (Labsystems, Finland). The experiments were performed in triplicate and at least repeated three times.

Cell cycle analysis

The effect of luteolin treatment on cell cycle progression was determined by flow-cytometric analysis. DNA content was assessed by staining ethanol-fixed cells with propidium iodide. AGS cells (1×10^6 cells/ml) were seeded in T75 flasks, and allowed to attach overnight. The medium was replaced with completely fresh medium containing desired concentrations of luteolin or control (0.5% DMSO). After incubation for indicated time intervals at 37°C, cells were washed with PBS and fixed in 70% ethanol for at least 12 h. The cells were then treated with 100 μ g/ml RNase A and 50 μ g/ml propidium iodide for 30 min. Stained cells were monitored by the FACSCalibur (BD Biosciences). And the percentages of cells within the G1, S, and G2/M phases of cell cycle were determined using a MultiCycle program (Phoenix Flow Systems, San Diego, CA).

Apoptosis analysis

The apoptosis-inducing effect of luteolin was investigated by two methods: (i) flow-cytometric analysis of cells with subdiploid DNA content and (ii) quantification of phosphatidylserine residues with Annexin V-FITC (BioVision, California, USA) and propidium iodide double staining kit. For the latter assay, cells were stained simultaneously with FITC-conjugated Annexin V and propidium iodide to enable the discrimination of intact cells (Annexin V⁻ PI⁻), early apoptotic cells (Annexin V⁺ PI⁻), and late apoptotic or necrotic cells (Annexin V⁺ PI⁺). Cells were seeded in a six-well plate at 1×10^6 cells/ml and incubated overnight. After 72 h luteolin treatment, cells were trypsinized, and washed with cold PBS and fixed with 70% ethanol at 4°C. Then the cells were washed with PBS again and incubated at room temperature with staining solution for 1 h in the dark. Stained cells were analyzed by the FACSCalibur flow cytometer (BD Biosciences) ($E_x = 488$ nm, $E_m = 530$ nm) and the results are indicated by a percentage of total gated cells (10^4 cells).

Extraction of cytosol and mitochondria lysate

To determine cytochrome C translocation, cytosolic and mitochondrial extracts were prepared from cells that had undergone drug treatment. Cells were treated and harvested as described above, suspended in buffer A (20 mM HEPES-KOH (pH 7.5), 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, and 250 mM sucrose) for 30 min on ice, and sonicated five times during this period. After centrifugation for 10 min at

3,500g, the supernatants were collected and further centrifugated for 15 min at 1,400g. The later supernatant was the cytosol lysate, and the pellet was suspended in protein extraction buffer and centrifugated again to obtain the mitochondrial lysate.

Western blot analysis

Gastric cancer cells were incubated in the presence of luteolin for 72 h. Cells were then rinsed twice with ice-cold PBS and lysed in protein lysis buffer (20 mM sodium phosphate, 150 mM sodium chloride, 1% Triton X-100, 5 mM EDTA, 5 mM phenylmethylsulfonyl fluoride, 1% aprotinin, 1 µg/ml leupeptin, 500 µM Na₃VO₄). Proteins were subjected to electrophoresis on 10% polyacrylamide gels and then transferred to a nitrocellulose membrane (Millipore, Bedford, MA) by electrotransfer. After blocking with 5% dried nonfat milk in PBS buffer and 0.5% Tween 20, the membrane was probed with various antibodies against Caspase-3, 6, 8, 9, Cdc2, Cyclin B1, Cdc25C, p21/cip1, p27/kip1, Bcl-xL, Bcl-2, Bax, p53, and cytochrome C, respectively. Membranes were then washed and incubated with the appropriate secondary antibodies. Finally, immunoreactive proteins were visualized with enhanced chemiluminescence (Amersham International, Buckinghamshire, UK).

Drug combination study

The inhibition effects on AGS cells of cisplatin, 5-Fu, gemcitabine, doxorubicin, and paclitaxel alone, and their combinations with luteolin, were determined by the MTT assay. The combination index (CI) was calculated by the Chou–Talalay equation [15], which takes into account both the potency (D_m or IC_{50}) and the shape of the dose–effect curve. The general equation for the classic isobologram ($CI = 1$) is given by $CI = (D)_1/(D_x)_1 + (D)_2/(D_x)_2 + [(D_x)_1 \cdot (D_x)_2]/[(D)_1 + (D)_2]$, where $(D_x)_1$ and $(D_x)_2$ in the denominators are the doses (or concentrations) for D_1 (drug 1) and D_2 (drug 2) alone that gives $x\%$ inhibition, whereas $(D)_1$ and $(D)_2$ in the numerators are the doses of drug 1 and drug 2 in combination, which also inhibited $x\%$ (i.e. isoeffective). $CI < 1$, $CI = 1$, and $CI > 1$ indicate synergism, additive, and antagonism effect, respectively [15, 16].

Statistic analysis

All analyses were performed using the SAS 6.12 software (SAS Institute, Inc., Cary, NC). Results are expressed as mean \pm SD. The two-sided t test was used to compare the difference between treatment groups. Differences were considered significant if the p value is less than 0.05.

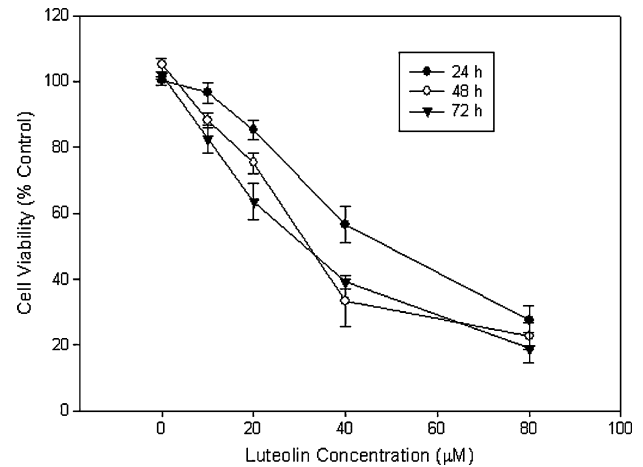


Fig. 1 The anti-proliferative effect of luteolin in AGS cells. Cells were treated with indicated concentrations of luteolin (µM) for 24, 48, and 72 h. Viability was determined using the MTT assay

Results

Luteolin inhibited the cell viability

To study whether luteolin inhibits AGS cell growth, we examined the cytotoxic effects of serial concentrations (0–80 µM) of luteolin for 24, 48, and 72 h treatments using the MTT assay. As shown in Fig. 1, luteolin significantly inhibited cell growth in a dose-dependent and time-dependent manner. Compared with untreated cells, the viability of cells treated with luteolin had a 50% decrease after a 24-h treatment with 50 µM luteolin. Moreover, cell growth was inhibited to 22% and 19% of controls with 80 µM luteolin at 48 and 72 h, and the IC_{50} values were 29.6 ± 3.8 (48 h) and 23.5 ± 2.4 µM (72 h).

Luteolin induced cell cycle arrest

Because the inhibition of proliferation by luteolin could be the result of cell cycle arrests or apoptosis or both, we then examined the effects of different concentrations of luteolin on cell cycle progression after 72 h treatment. As shown in Fig. 2a, luteolin treatment resulted in a dose-dependent accumulation of cells in G2/M phase. Concomitantly, there was a significant decrease of cells in the S phase. At the dose of 80 µM luteolin, the percentage of cells in G2/M phase reached 57% of total cells, compared to 4.98% in the control group ($p < 0.01$), whereas the percentage of cells in S phase decreased to 17.2%, compared to 48.31% in the control group ($p < 0.01$) (Fig. 2b). These data indicated that luteolin promotes G2/M arrest in AGS cells.

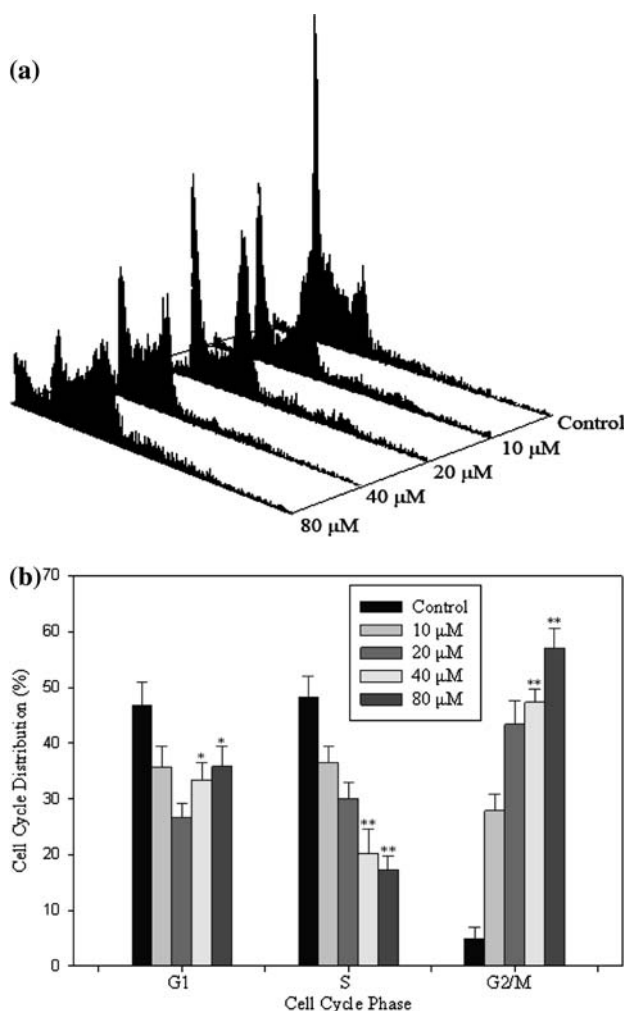


Fig. 2 Luteolin induced cycle arrest in AGS cells. Luteolin was added at the indicated concentrations and cells were incubated for 72 h. Cell cycle proportions were determined by flow cytometry after staining with propidium iodide. **(a)** Flow cytometry of cell cycle phase distribution; **(b)** Statistical analysis of cell cycle phase distribution. Each value represents the mean of three experiments; bars, \pm SD. (* $p < 0.05$, ** $p < 0.01$)

Luteolin decreased Cdc2, Cyclin B1, and Cdc25C levels, and increased p21/cip1 level

It is known that the progression through various phases of the cell cycle is promoted by Cyclins and Cdks and inhibited by Cdk inhibitors [17]. Since luteolin treatment resulted in G2/M arrest in AGS cells, we then assessed their effects on the expression of proteins that regulate the G2/M phase transition, including Cdc2, Cyclin B1, Cdc25C, p21/cip1, and p27/kip1. When the cells were treated with luteolin of different concentrations for 72 h, a marked dose-dependent decrease of Cdc2, Cyclin B1, and Cdc25C levels were observed using western blot analysis (Fig. 3). Conversely, the expression of p21/cip1 increased in a dose-dependent manner, but the expression of p27/kip1 had no apparent change (Fig. 3).

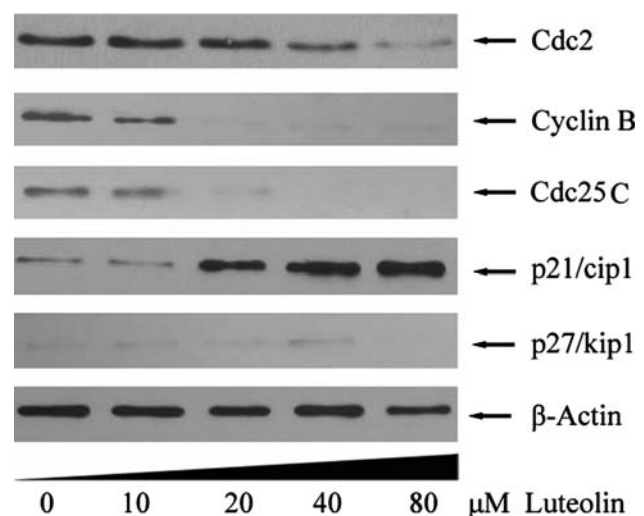


Fig. 3 Luteolin altered the expression of cell cycle related proteins, Cdc2, Cyclin B1, Cdc25C, p21/cip1, and p27/kip1. The expression of proteins was assessed by immunoblotting. The AGS cells treated with various concentrations of luteolin for 72 h

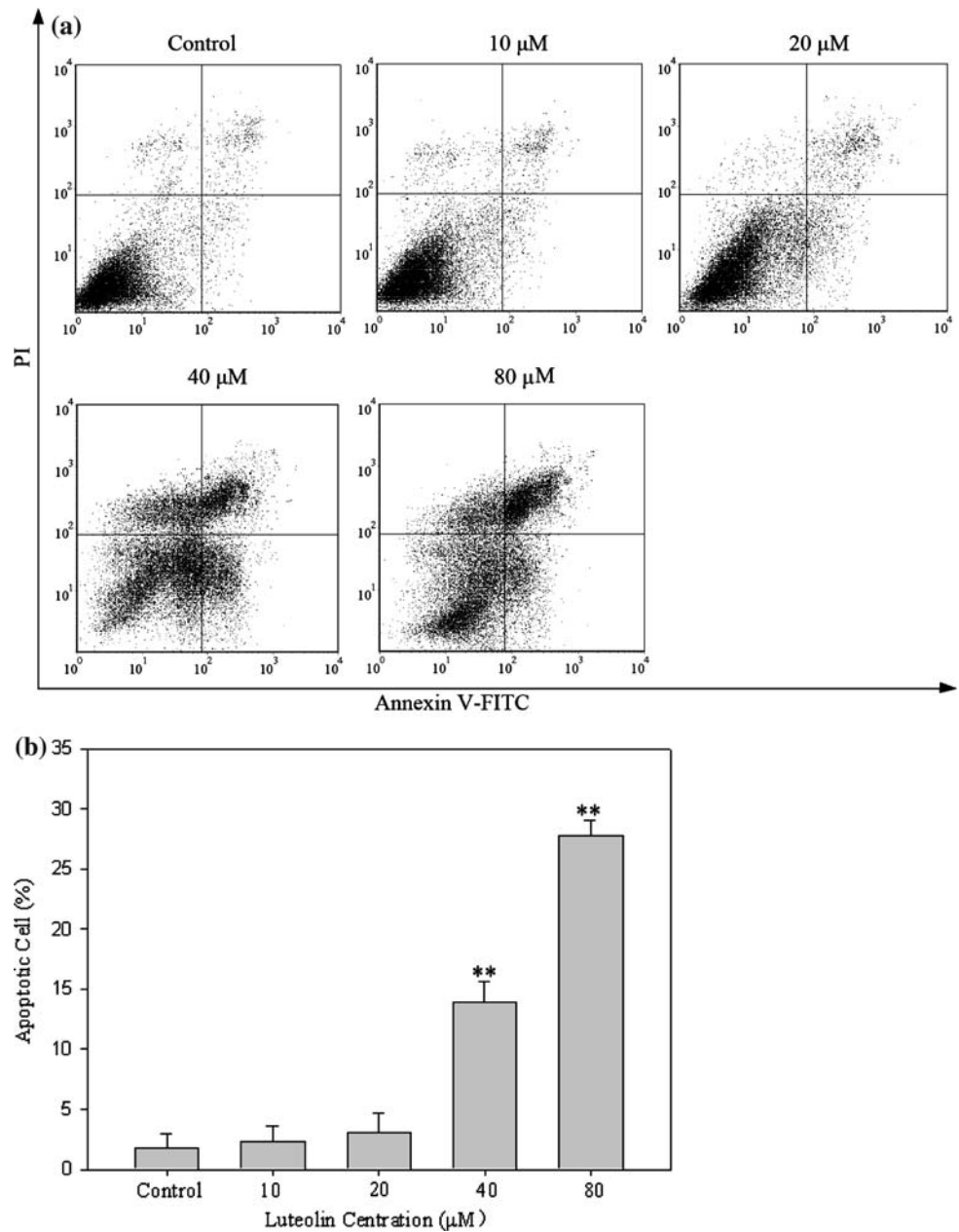
Luteolin induced apoptosis

Upon treatment of AGS with luteolin, the accumulation of a sub-G₁ peak of hypodiploid cells was evident in a concentration-dependent manner, whereas the proportion of the sub-G₁ peak was negligible in control cells (Fig. 2a). At the concentration of 80 μM, the percentage of cells in sub-G₁ phase reached 22.7% of total cells, indicating the induction of apoptosis. Annexin V staining detects early apoptosis by binding to phosphatidylserine on membrane, which is translocated from the inner to the outer leaflet of the plasma membrane during apoptosis (Fig. 4a). Moreover, double staining with Annexin V and propidium iodide examined cells in the late stage of apoptosis. As shown in Fig. 4b, the percentage of total apoptotic cells were increased to 13.91% and 27.73% after the treatment with 40 μM and 80 μM luteolin, respectively, which were significantly higher than that (1.77%) in control cells ($p < 0.01$). Similarly, the number of early apoptotic cells with the treatment of 40 and 80 μM luteolin were also much higher than that of the control cells (16.08% and 14.26% vs. 1.44%, respectively).

Effects of luteolin on the levels of pro-apoptotic proteins and anti-apoptotic proteins

Apoptosis is regulated by a variety of pro-apoptotic proteins and anti-apoptotic proteins [18–20]. We examined the expression levels of some apoptosis regulatory proteins, including p53, Bcl-2, Bak, Bax, Bcl-xL, Caspase-3, 6, 8, 9, and PARP. As shown in Fig. 5a, western blot showed that the protein levels of the active subunits of Caspase-3, 6,

Fig. 4 Luteolin induced apoptosis in AGS cells. Luteolin was added at the indicated concentrations and cells were incubated for 72 h. Apoptosis was assessed by Annexin V binding assay. **(a)** Flow-cytometric analysis; **(b)** Statistical analysis for apoptosis. Each value represents the mean of three experiments; bars, \pm SD. (* $p < 0.05$, ** $p < 0.01$)



and 9 were elevated in a luteolin dose-dependent manner, but no changes of Caspase-8 was found. PARP degradation also supported the observation of Caspase activation (Fig. 5a). Similarly, the levels of pro-apoptotic proteins Bax and p53 were increased, but no change was detected for Bak. Conversely, the level of anti-apoptotic protein Bcl-2 was decreased, but no change was found for Bcl-xL (Fig. 5b). Furthermore, Fig. 5c showed that luteolin treatment resulted in a significant shift of cytochrome C from the mitochondrial fraction to the cytosolic fraction, which supported an activation of the intrinsic apoptotic pathway rather than the extrinsic pathway.

Synergic effect of luteolin and cisplatin

Cisplatin is one of the most commonly used therapeutic agents for gastric cancer, via its direct interaction with DNA to form bulky DNA adducts. In this study, we examined the chemosensitizing effects of luteolin on cisplatin in AGS cells. Cells were treated for 72 h with cisplatin alone or in combination with luteolin at a ratio of 1:1 for several different doses. It demonstrated that the fraction of cell growth inhibition increased more evidently with a combinational treatment of cisplatin and luteolin, compared to cisplatin treatment alone (Fig. 6a). The CI/fractional effect plots

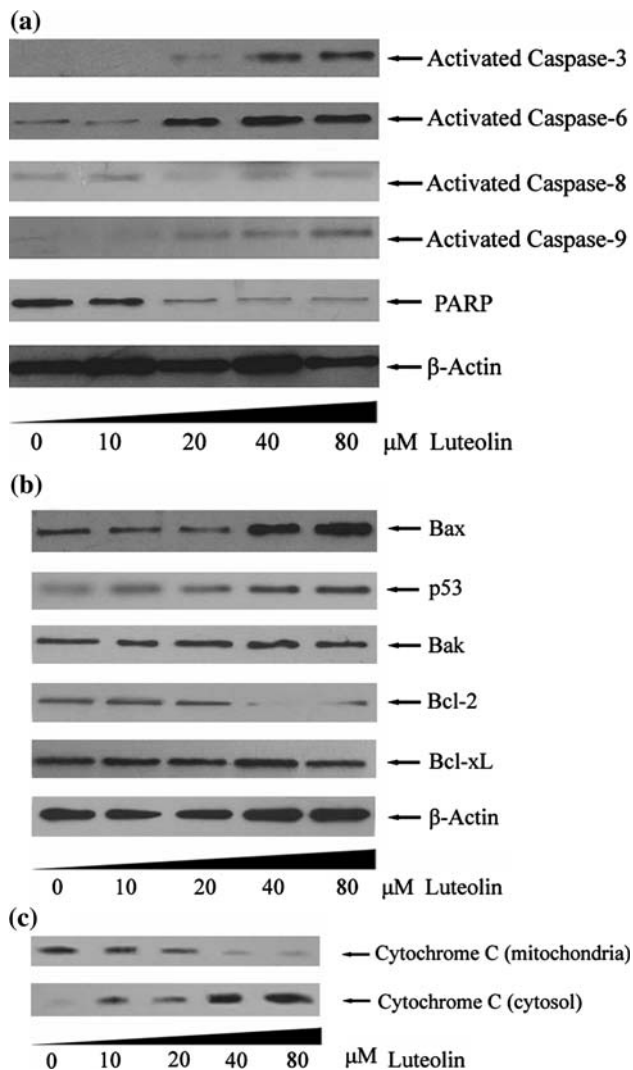


Fig. 5 Luteolin altered the expression of apoptosis related proteins. (a) Caspase-3, 6, 8, 9, and PARP; (b) p53, Bcl-2, Bak, Bax, and Bcl-xL; (c) translocation of cytochrome C. The expression of proteins was assessed by immunoblotting. The AGS cells were treated with various concentrations of luteolin for 72 h

showed the CI values versus the fraction of cells affected by cisplatin and luteolin in combination, and there was a synergistic ($\text{CI} < 1$) effect for the two agents on AGS cells (Fig. 6b). However, luteolin cannot sensitize AGS cells to other chemotherapeutic agents, like 5-Fu, gemcitabine, doxorubicin, and paclitaxel (data not shown).

Discussion

Conventional chemotherapy of gastric cancer has its limitations on improving the treatment outcomes and quality of survival/life in human patients; so prevention and therapeutic intervention by dietary and nondietary phytochemicals are newer approaches as compared to the use of cytotoxic

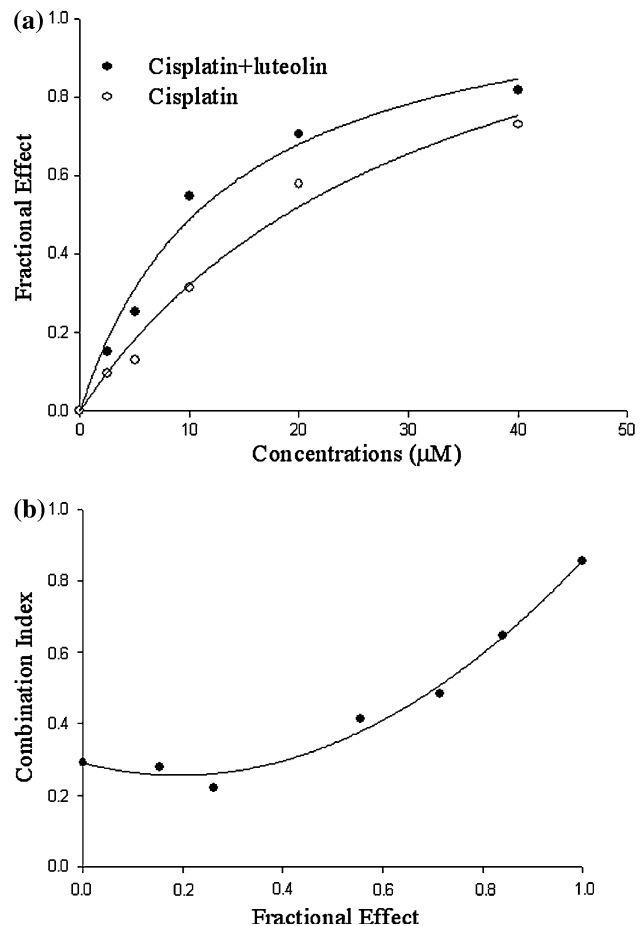


Fig. 6 Combination effects of luteolin and cisplatin. (a) AGS cells were treated for 72 h with cisplatin and luteolin alone or in combination at a ratio of 1:1 for several different doses. MTT assays were performed to measure the fractional effect of cell growth inhibition. (b) The CI/fractional effect plots showed the CI values versus the fraction of cells affected by cisplatin and luteolin in combination. Synergy between luteolin and cisplatin was calculated from median effect plots of (a). CI values below 1 represent synergy

chemotherapeutic agents in cancer management [1–3]. Luteolin, a plant flavonoid, has been shown to have anti-carcinogenic properties in some cancer cell lines [8]. In the present study, we have shown that luteolin inhibit the growth of gastric cancer AGS cells through the following mechanisms: (a) luteolin promotes G2/M arrest and regulates the expression of different enzymes related to G2/M transition and (b) luteolin induces apoptosis and affects the levels of both pro-apoptotic and anti-apoptotic proteins, especially those in the intrinsic apoptotic pathway. In addition, luteolin sensitizes AGS cells to cisplatin and has synergistic effect with cisplatin on AGS cells growth inhibition.

Previously, luteolin has been known to suppress tumor cell growth through both cell cycle arrest and apoptosis [13, 21–23]. Our results in gastric cancer cell lines were consistent with those findings and showed that luteolin suppress the growth of AGS cells through blocking the cell

cycle progression at G2/M phases and activating intrinsic apoptotic pathway. In terms of the regulation of cell cycle, Cyclin-dependent kinases (CDK) and Cyclin-dependent kinases inhibitors (CDKIs) play important roles. In G2 and M phases, cdc2 kinase is activated by binding to Cyclin B. Cdc25C plays a critical role in the dephosphorylation of Cdc2 on Thr14/Tyr15, and the blockage of its activity inhibits the subsequent activation of Cyclin B1/Cdc2. In this study, luteolin was found to down-regulate Cdc2, Cyclin B1, and Cdc25C protein levels, which were required for the progression of G2/M checkpoints. p21/cip1 and p27/kip1 are two important members of CDKIs that inhibits the activity of CDK-Cyclin complexes and negatively regulate cell cycle progression. It has been showed that p21/cip1 regulated G1 and S progression by inhibiting cdk4 and cdk2 activities [24]. However, some later studies suggested that p21/cip1 was also involved in the suppression of G2/M transition [25, 26]. Our results showed that luteolin increased p21/cip1 protein levels, but not p27/kip1. All these results indicated that the regulation of G2/M transition may be an effective target to control the growth and proliferation of AGS cells by luteolin.

Impaired apoptosis is another crucial mechanism for cancer development. The Bcl-2 family proteins are critical in the conduction of apoptosis and can be divided into pro- and anti-apoptotic members [27]. Studies have shown that anti-apoptotic Bcl-2 family proteins form a heterodimer with Bax/Bak and thereby suppress the pro-apoptotic effects [28]. In addition, Bcl-2 family proteins regulate the release of cytochrome C from the mitochondria into cytosol, which will activate initiator caspase-9 and induce a subsequent caspase cascade (the intrinsic apoptotic pathway) [29]. In this study, we have showed that luteolin treatment significantly increased the levels of pro-apoptotic proteins, including Caspase-3, 6, 9, Bax, and p53, and decreased the levels of anti-apoptotic Bcl-2 protein, thus shifting the Bax/Bcl ratio in favor of apoptosis. The levels of cytochrome C did not change when whole-cell extracts were analyzed (data not shown). However, subcellular translocation of Cytochrome C was observed, which further supported the conclusion that luteolin treatment induced apoptosis in AGS cells through an intrinsic mitochondria apoptotic pathway.

The resistance of tumor cells to cytotoxic agents is one of the major limitations in cancer chemotherapy; therefore combinational treatment with sensitizing agents, such as luteolin, may be an effective strategy to overcome cisplatin resistance [30, 31]. Shi et al. [29] found that luteolin enhanced the cisplatin-induced apoptosis in some human cancer cells and thereby sensitized the anti-cancer effect of cisplatin. In the present study, our results showed that the combination of cisplatin and luteolin was more effective in inhibiting AGS cell growth, indicating an application

potential of luteolin as a chemosensitizer in gastric cancer therapy.

In conclusion, luteolin can significantly inhibit the proliferation of gastric cancer cells through both G2/M arrest and enhanced apoptosis, and has a synergistic effect with cisplatin on the suppression of gastric cancer cell growth. Although further investigations are warranted to evaluate the anti-cancer effects of luteolin, data from this study provide the clinical application potential for luteolin in gastric cancer chemotherapy.

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