RESEARCH ARTICLE

Luteolin Arrests Cell Cycling, Induces Apoptosis and Inhibits the JAK/STAT3 Pathway in Human Cholangiocarcinoma Cells

Ploypailin Aneknan[1], Veerapol Kukongviriyapan[1,2], Auemduan Prawan[1,2], Sarinya Kongpetch[1,2], Banchob Sripa[3], Laddawan Senggunprai[1,2]*

Abstract

    Cholangiocarcinoma (CCA) is one of the aggressive cancers with a very poor prognosis. Several efforts have been made to identify and develop new agents for prevention and treatment of this deadly disease. In the present study, we examined the anticancer effect of luteolin on human CCA, KKU-M156 cells. Sulforhodamine B assays showed that luteolin had potent cytotoxicity on CCA cells with IC50 values of 10.5±5.0 and 8.7±3.5 µM at 24 and 48 h, respectively. Treatment with luteolin also caused a concentration-dependent decline in colony forming ability. Consistent with growth inhibitory effects, luteolin arrested cell cycle progression at the G2/M phase in a dose-dependent manner as assessed by flow cytometry analysis. Protein expression of cyclin A and Cdc25A was down-regulated after luteolin treatment, supporting the arrest of cells at the G2/M boundary. Besides evident G2/M arrest, luteolin induced apoptosis of KKU-M156 cells, demonstrated by a distinct sub-G1 apoptotic peak and fluorescent dye staining. A decrease in the level of anti-apoptotic Bcl-2 protein was implicated in luteolin-induced apoptosis. We further investigated the effect of luteolin on JAK/STAT3, which is an important pathway involved in the development of CCA. The results showed that interleukin-6 (IL-6)-induced JAK/STAT3 activation in KKU-M156 cells was suppressed by treatment with luteolin. Treatment with a specific JAK inhibitor, AG490, and luteolin diminished IL-6-stimulated CCA cell migration as assessed by wound healing assay. These data revealed anticancer activity of luteolin against CCA so the agent might have potential for CCA prevention and therapy.

Keywords: Luteolin - cholangiocarcinoma - cell cycle - apoptosis - JAK/STAT3 - migration

Introduction

    Cholangiocarcinoma (CCA) is a tumor originating from the epithelial cells of the biliary tract. It is a rare type of cancer worldwide, however, populations residing in the Southeast Asian region are at a very high risk. The high incidence of CCA in this region is associated with background conditions, particularly liver fluke infection (i.e. Opisthorchis viverrini and Chlonorchis sinensis) that causes long-standing inflammation, cell injury, and reparative biliary epithelial cell proliferation (Sripa et al., 2012). Persistent inflammation can create a local environment enriched with cytokines and other growth factors (Sripa et al., 2012). The activation of the intracellular JAK/STAT3 signaling pathway triggered by these inflammatory mediators leads to the induction of genes involved in the development of CCA (Prakobwong et al., 2011). The pathway, therefore, is gaining attention as a molecular target for CCA treatment strategies.

    It is known that one of the causes of cancer including CCA is uncontrolled proliferation. Cell proliferation is governed by the cell cycle, which is an order of events that is tightly regulated by a number of serine/threonine protein kinases known as cyclin-dependent kinase (CDK) and cyclins (Lim and Kaldis, 2013). In addition, the cell division cycle 25 (Cdc25) phosphatases are also a family of proteins that are vital to cell cycle regulation (Lavecchia et al., 2012). Furthermore, cancer cells acquire alternations for enhanced survival and become apoptosis-resistant to anticancer therapies. Apoptosis is a form of programmed cell death. It has been considered as the major form of cell death in various physiological events (Sankari et al., 2012). Perturbing cell cycle progression can cause severe damage to cells and may trigger apoptosis (Sankari et al., 2012). The induction of the cell cycle arrest and apoptosis can be an effective approach to inhibit uncontrolled cell proliferation and survival of malignant tumor cells.

    Currently, radical surgery is the optimal therapy for CCA with curative potential. Most patients, however, present with advanced disease at the time of diagnosis and these patients are often deemed poor candidates for curative surgery (Mihalache et al., 2010). Chemotherapy

[1]*Department of Pharmacology, Faculty of Medicine,* [2]*Liver Fluke and Cholangiocarcinoma Research Center,* [3]*Department of Pathology, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand* *For correspondence: laddas@kku.ac.th*

is a remaining option, however, current chemotherapeutic drugs have shown disappointing results in terms of survival benefit in inoperable patients (Butthongkomvong et al., 2013). Hence, the development of new chemotherapeutic agents is highly needed.

Dietary polyphenols from natural sources are known to possess several important therapeutic effects, importantly, cancer chemoprevention and cancer chemotherapeutic activities (Ali et al., 2012). Luteolin (3', 4', 5, 7-tetrahydroxyflavone) is a common dietary flavonoid found in a variety of vegetables, fruits and medicinal herbs including celery, parsley, broccoli, carrots, cabbage, green peppers, apples and chamomile tea (Lopez-Lazaro, 2009). Luteolin has been shown to be beneficial to human health due to its strong antioxidant, radical scavenging and anti-inflammatory properties (Lopez-Lazaro, 2009). Moreover, there are several reports about the anticancer properties of luteolin including induction of cancer cell apoptosis and cell cycle arrest (Wang et al., 2012), and inhibition of cancer cell invasiveness (Ruan et al., 2012) and angiogenesis (Pratheeshkumar et al., 2012). Despite several studies elucidating significant anticancer activity of luteolin against various cancers (Wang et al., 2012; George et al., 2013), its activity against CCA remains to be addressed. The purpose of this study was to investigate the anticancer effect of luteolin on human CCA cells and to gain insights regarding the underlying mechanism mediating its effects. The results demonstrate the potent anticancer activity of luteolin and this may provide a new approach to the CCA therapy.

Materials and Methods

Materials

Luteolin, sulforhodamine B (SRB), acridine orange, ethidium bromide and propidium iodide (PI) were obtained from Sigma Chemical (St. Louis, MO, USA). AG490 was obtained from Calbiochem (San Diego, CA, USA). Interleukin-6 (IL-6) was purchased from Prospec-Bio (Israel). RIPA Lysis Buffer was obtained from Amresco (Solon, OH, USA). The primary antibodies against STAT3 and phospho-STAT3 (Tyr705) were purchased from Cell Signaling Technology (Dancers, MA, USA). The primary antibodies against Bcl-2, cyclin A, Cdc25A, β-actin and the secondary horseradish peroxidase (HRP)-linked antibodies were obtained from Santa Cruz Biotechnology, Inc. (California, USA). The Amersham™ ECL™ Prime Western Blotting Detection Reagent was from Amersham Biosciences Corp. (NJ, USA). Reagents for cell culture were from Gibco BRL Life Technologies (Grand Island, NY, USA).

Cell line and cell culture

The human CCA cell line KKU-M156 used in this study was kindly provided by Dr. Banchob Sripa of Department of Pathology, Faculty of Medicine, Khon Kaen University. This cell line was cultured in complete media consisting of Ham's F12 media, supplemented with 10% fetal calf serum, 12.5 mM HEPES, pH 7.3, 100 U/ml penicillin G and 100 µg/ml streptomycin and maintained under an atmosphere of 5% CO_2 at 37°C. The cells were subcultured every 2 days using 0.25% trypsin-EDTA.

Cell viability assay

SRB was used to measure the effect of luteolin on the viability of CCA cell lines as described previously (Prawan et al., 2009). Briefly, KKU-M156 cells were plated in a 96-well plate for 24 h. After exposure of cultured cells to luteolin at various concentrations for 24 or 48 h, the cultured cells were fixed with ice-cold trichloroacetic acid and stained with 0.4% SRB in 1% acetic acid. Excess dye was removed by rinsing several times with 1% acetic acid, and protein-bound dye was dissolved with 10 mM Tris base solution for determination of absorbance with a microplate reader with a filter wavelength of 570 nm. Cell growth inhibition was expressed in terms of percentage of untreated control absorbance following subtraction of mean background absorbance. The IC_{50} concentration (50% inhibition of cell growth values) was calculated from the dose-response curves.

Clonogenic assay

The assay was carried out as a method described previously with minor modifications (Hu et al., 2013). The 800 viable cells were seeded in 6 well plates and allowed to grow for 24 h. The cells were then treated with different concentrations of luteolin for 24 h. The cells were washed with PBS and fresh medium was provided. The cells were grown for another 8 days. The cultured cells were stained with crystal violet, and colonies were viewed and counted.

Cell cycle analysis

KKU-M156 cells were treated with luteolin at different concentrations of 5, 10 and 50 µM for 48 h. Then cells were collected and fixed in 70% cold ethanol at -20°C overnight. After washing twice with PBS, cells were resuspended in PBS. RNase A (0.02 mg/ml) and propidium iodide (PI) (0.02 mg/ml) were added to the fixed cells for 1 h at 4°C. The DNA content of cells was then analyzed with a flow cytometer (BD Biosciences, San Jose, CA). The percentage of cells in the different cell cycle phases was calculated using BD FACSDiva™ Software (BD Biosciences, San Jose, CA).

Acridine orange / ethidium bromide (AO/EB) staining

AO/EB staining was used to determine apoptotic and necrotic cells. Briefly, at the end of the treatment with luteolin for 48 h, cultured cells were rinsed with PBS and stained with AO and EB (each 1 µg/ml). The fluorescent images were captured using a Nikon Eclipse TS100 inverted microscope with excitation and long-pass emission filters of 480 and 535 nm. The numbers of viable, apoptotic and necrotic cells were enumerated and calculated as the percent cells over a total number of cells in the same area as previously described (Suphim et al., 2010).

Protein extraction and western blot analysis

After treatments for designated period of times, medium was aspirated, cells were washed twice with cold PBS and whole cell lysates were prepared using RIPA cell lysis buffer according to the manufacturer's instructions.

The 20 μg of proteins were resolved by SDS-PAGE using 10% polyacrylamide gel and the separated proteins on the gel were electrophoretically transferred to a PVDF membrane. The membranes were blocked with 5% (w/v) bovine serum albumin in phosphate buffered saline containing 0.1% Tween-20 for 2 h at room temperature, followed by incubation with the primary antibodies against STAT3 (1:2000), phospho-STAT3 (Tyr705) (1:1000), cyclin A (1:1000), Cdc25A (1:1000), Bcl-2 (1:2500) and β-actin (1:5000) at 4°C overnight. After washing, the membranes were incubated with the appropriate horseradish peroxidase conjugated secondary antibody to detect bands by Amersham™ ECL™ Prime. The densities of the specific protein bands were visualized and captured by ImageQuant™ 400.

Wound healing assay

Cell migration was assessed using a wound healing assay as a method described previously (Senggunprai et al., 2013). Briefly, KKU-M156 cells (1.5 x 10⁵) were seeded into a 24-well plate and incubated overnight in Ham's F12 medium, supplemented with 10% fetal calf serum. A scratch wound was made with a sterile 200 μL pipette tip. After washing with PBS to remove any detached cells, a series of images of the scratched wound were taken from 0 to 36 h. The cells were pretreated with AG490, a specific JAK inhibitor, at 10 μM, luteolin at 0.3 and 3 μM or a vehicle for 2 h, and then with 200 ng/ml of IL-6 afterward. The closing of scratched wound, represented the migration process, was determined by capture of the denuded area along the scratch using Image-Pro Plus software (Media Cybernetics, LP, USA). The wound distance was calculated by dividing the area by the length of scratch.

Statistical analysis

Statistical comparison between the control and treatment groups was performed with the Student's t-test. Results were considered to be statistically significant at $p<0.05$. The IC_{50} values calculations and statistical analyses were performed using the Prism 5 program (GraphPad Software, San Diego, CA, USA).

Results

Luteolin decreased CCA cell viability

The anticancer effect of luteolin was firstly evaluated by examining its effect on the growth of CCA KKU-M156 cells using the SRB assay. The results showed that the cell growth was strongly inhibited after 24 and 48 h (Figure 1A) of treatment with luteolin in a dose-dependent manner with the IC_{50} values of 10.5±5.0 and 8.7±3.5 μM, respectively.

Luteolin inhibited colony formation efficacy

To determine the effect of luteolin on longer term viability and replicative potential of CCA cells, the clonogenic assay was performed. Treatment with luteolin caused a concentration-dependent decline in colony forming ability of KKU-M156 cells (Figure 1B).

*Luteolin arrested CCA cell cycle progression*

To assess whether luteolin-induced CCA cell growth inhibition is mediated via alterations in cell cycle progression, luteolin's effects on the cell cycle phase distribution in KKU-M156 cells were determined using flow cytometry with PI staining. Consistent with growth inhibitory effects, luteolin caused a significant increase in the distribution of cells at the G2/M phase in a dose-dependent manner. Besides evident G2/M arrest, distinctive sub-G1 peaks, which were recognized as the apoptotic fractions (Patel et al., 1998), were observed in KKU-M156 cells after treatment with 50 μM luteolin (Figure 2A). To further investigate the molecules affected by luteolin, the expression levels of cell cycle-associated proteins under the same conditions were examined. The results demonstrated that 50 μM luteolin treatment of cells resulted in a dramatic decrease in the protein levels of cyclin A as well as in Cdc25A (Figure 2B).

Luteolin induced apoptosis of CCA cells

To confirm that cell death affected by luteolin could involve apoptotic induction, individual apoptosis in the cell population of KKU-M156 cells treated with luteolin was assessed by the fluorescence staining (AO/EB) method. The results showed that 10 μM luteolin slightly induced apoptosis in KKU-M156 cells. Treatment with 50 μM luteolin, however, remarkably induced apoptosis in CCA cells (Figure 2C). Further investigation of the expression of a major protein responsible for regulating the mitochondrial apoptosis pathway, anti-apoptotic Bcl-2, was then initiated. Western blot analysis demonstrated that following treatment with luteolin at 50 μM, Bcl-2 expression was markedly down-regulated (Figure 2B).
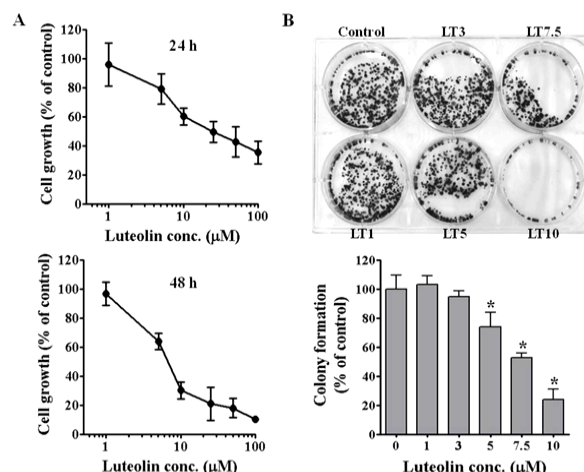
Figure 1. Effect of Luteolin on the Growth of CCA Cells. A) KKU-M156 cells were treated with various concentration of luteolin (1, 5, 10, 25, 50 and 100 μM) for 24 or 48 h. After treatment, the cell numbers were determined by the SRB assay. The results are presented as percentage of control. The data are the mean±SD averaged from three independent experiments. **B)** Cells were grown in 6-well plates and treated with indicated concentrations of luteolin. After 8-days, cells were stained with crystal violet and photographed. Figures shown are representative of four independent experiments. The graph shows the percentage of colony formation relative to the controls. Data represent mean±SD averaged from four independent experiments. LT, luteolin. *p<0.05 *vs* control
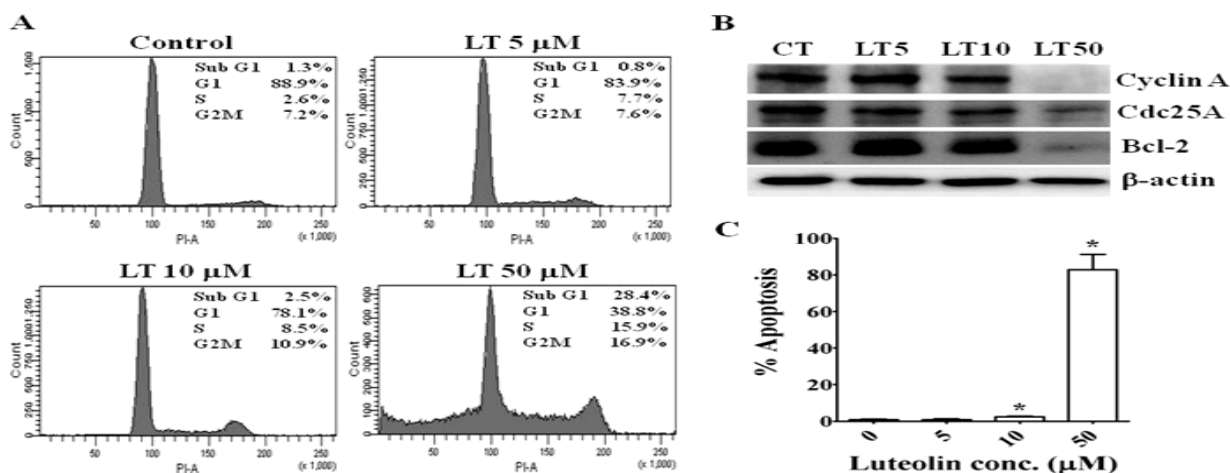
Figure 2. Effects of Luteolin on the Distribution within the Cell Cycle, Induction of Apoptosis and Expression of the Cell Cycle- and Apoptosis-Associated Proteins in CCA Cells. KKU-M156 cells were treated with various concentration of luteolin (5, 10 and 50 μM) for 48 h. **A)** The cells were stained with PI and analyzed by flow cytometry. Figures shown are one of two similar experiments. **B)** The cells were analyzed for the expression of cell cycle and apoptosis regulatory proteins using western blotting. Results shown are representative of two similar experiments. **C)** The cells were stained with fluorescent dye AO/EB and viewed under a microscope. The apoptotic cells were calculated as the percent apoptotic cells over a total number of cells in the same area. The data are the mean±SD averaged from three independent experiments (triplicate in each experiment). CT, control; LT, luteolin. *p<0.05 *vs* control
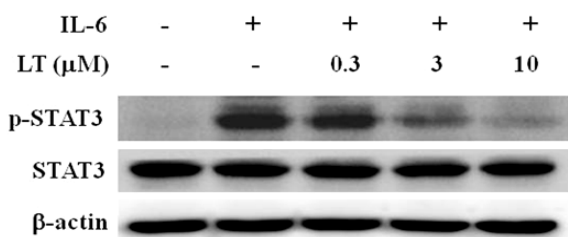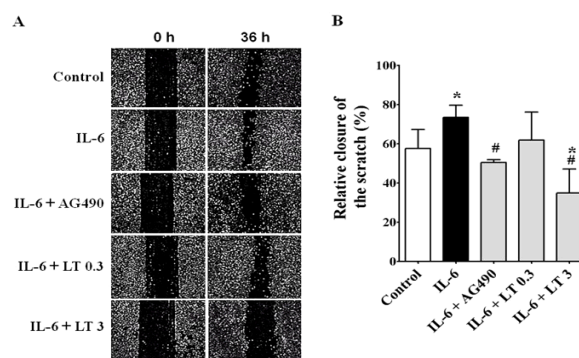

Figure 3. Effects of Luteolin on IL-6-Induced STAT3 Activation. KKU-M156 cells were pretreated with the indicated concentrations of luteolin and then treated with 200 ng/ml of IL-6 for 30 min. Whole cell extracts were prepared and subjected to western blot analysis using β-actin as the loading control. Results shown are representative of two similar experiments. LT, luteolin

Figure 4. Effects of AG490 and Luteolin on IL-6-Stimulated Migration of CCA Cells. A) Scratched wounds of monolayer KKU-M156 cells were pretreated with vehicle, 10 μM AG490 or luteolin (0.3 or 3 μM) for 2 h and then treated with 200 ng/ml of IL-6. Cell migration was monitored under phase-contrast microscopy (x4 magnification). Representative images of wound healing were obtained at the time of the scratch and 36 h later. **B)** The graph shows the level of cell migration into the wound scratch quantified as the percentage of wound closure at 36 h. The data are mean±SD averaged from three independent experiments (triplicate in each experiment). LT, luteolin. *p<0.05 *vs* control. #p<0.05 *vs* IL-6 alone.

Luteolin suppressed the JAK/STAT3 cascade of CCA cells

Because the JAK/STAT3 signaling pathway has been shown to be a critical signaling pathway in cholangiocarcinogenesis (Isomoto, 2009; Dokduang et al., 2013), in the present study, the effect of luteolin on this pathway was examined using western blot analysis. STAT3 is known to be activated by cytokine IL-6, and inhibition of IL-6-induced STAT3 signaling might be an important mechanism of anticancer compounds. In this experiment, KKU-M156 cells were pre-incubated with various concentrations of luteolin for 2 h and then stimulated with IL-6 for 30 min. Under these conditions, the cell viability was not affected (data not shown). As shown in Figure 3, when KKU-M156 cells were pretreated with luteolin, IL-6-induced STAT3 phosphorylation was suppressed in a dose-dependent manner, although STAT3 protein levels were not affected.

Luteolin diminished IL-6-mediated CCA cell migration

We have recently reported that STAT3 activation in CCA cells leads to the up-regulation of expression of intercellular adhesion molecule-1 (ICAM-1), which is an adhesion molecule involved in cell migration and invasion (Senggunprai et al., 2013). In this study, it was then explored as to whether the JAK/STAT3 pathway activation mediates the migration of CCA cells. As illustrated in Figures 4A and 4B, IL-6 stimulated the migration of KKU-M156 cells, evidently by the closure of the scratched wound. Treatment of cells with a specific JAK inhibitor, AG490, significantly suppressed the cytokine-mediated cell migration, suggesting a role of JAK in IL-6-mediated CCA cell migration via STAT3 activation. In separate experiments, it was confirmed that the dose of IL-6 and AG490 used in this experiment only slightly affected the cell viability (data not shown). We further determined the

effect of luteolin on IL-6-stimulated CCA cell migration. The results clearly demonstrated that luteolin significantly abolished the IL-6-dependent CCA cell motility, as the migration distance of cultured cells in luteolin treated groups was shorter than those of IL-6 treatment only (Figure 4A and 4B).

Discussion

One of the greatest challenges in the treatment of CCA is the resistance to cancer chemotherapy. Several efforts have been made to identify and develop new agents for the treatment and prevention of this deadly disease. Compounds from natural sources have received very wide attention due to their anticancer activities and possible lead novel anticancer compounds. In the present study, it has been demonstrated that there are possible anticancer activities of luteolin in human CCA cells. Luteolin can inhibit the proliferation, arrest cell cycle progression and induce apoptosis of KKU-M156 cells, accompanied with suppression of metastatic potential mediated by the JAK/STAT3 signaling pathway. Hence, luteolin is a possible potential natural compound for use to prevent and as a treatment of CCA.

Since carcinogenesis is characterized by unregulated clonal expansion of malignant cells, one of the requisite actions of an anticancer agent is to impede the uncontrollable proliferation and accelerate death of tumor cells. In this study, it was found that luteolin has a remarkable effect in the induction of CCA cells death with the IC_{50} value in the low micromolar range. The ability of luteolin to inhibit cell growth in CCA seemed to be much more potent when compared to the effects on other types of cancers as reported by other investigators (Wang et al., 2012; George et al., 2013). To examine the mechanism responsible for cell growth inhibition, cell cycle distribution was evaluated using flow cytometry. The loss of the proliferative capacity of CCA cells treated by luteolin was associated with the G2/M phase arrest. Similar to these observations, previous studies found that luteolin blocked human colon and breast cancer cells at G2/M phase (Lee et al., 2012; Pandurangan et al., 2013). The effect of luteolin on a human esophageal squamous carcinoma cell line, however, was found to be arresting cell cycle progression at G0/G1 phase (Wang et al., 2012). The cell cycle in eukaryotic cells is regulated by expression and sequential activation of cell cycle-dependent cyclins, CDKs and CDK inhibitors (Lim and Kaldis, 2013). Cyclin A binds and activates CDK2, and thus promotes both cell cycle G1/S and G2/M transitions (Lim and Kaldis, 2013). In addition, Cdc25 phosphatases are also central targets and regulators of the G2/M checkpoint mechanisms (Lavecchia et al., 2012). The present results showed that protein expression of cyclin A and Cdc25A were down-regulated after the treatment of luteolin, supporting the arrest of cell cycle progression at the G2/M phase.

Additionally, luteolin also induced apoptosis, based on the sub-G1 group demonstrated in the cell cycle analysis and the results obtained from fluorescent dye staining. Apoptosis is a tightly regulated process, which involves changes in the expression of a distinct set of genes (Sankari et al., 2012). One of the major genes responsible for regulating the mitochondrial apoptosis pathway is anti-apoptotic Bcl-2 (Czabotar et al., 2014). In this study, pronounced apoptotic cells found in CCA cells treated with luteolin were accompanied with the decrease in Bcl-2 protein expression. These results indicate that the mechanisms of the growth inhibitory effect of luteolin on CCA cells are through cell cycle arrest and apoptosis induction, at least at the concentrations observed.

Cell signaling pathways have been become the targets for many novel anticancer agents. Among them, the JAK/STAT3 pathway is gaining more attention for cancer chemotherapy. STAT3 is known as an oncogene involved in the development of several cancers including CCA (Isomoto, 2009; Dokduang et al., 2013). In the present study, it was found that the JAK/STAT3 cascade of CCA cells was suppressed by luteolin. Indeed, luteolin has been shown to promote the degradation of STAT3 in human hepatoma cells (Selvendiran et al., 2006). Additionally, luteolin has been shown to exert its effects through inhibition of several signaling pathways such as AMPK and NF-ϰB as demonstrated in hepatocarcinoma cells (Hwang et al., 2011). The results obtained from this study suggest that the JAK/STAT3 pathway is a target for luteolin action in CCA cells. Further studies are required to elucidate its action on the other signaling pathways of CCA cells.

Metastasis is the most devastating aspect of cancer and remains a major cause of poor prognosis and death in cancer patients. It is composed of a cascade of interrelated and sequential steps, including cell adhesion, extracellular matrix degradation, cell movement and invasion (Hagedorn and Sherwood, 2011). In fact, a compound with the ability to block the metastasis-associated steps could be a potential candidate for cancer chemoprevention and chemotherapy. It has been reported that the activation of STAT3 modulates the transcription of a variety of genes involved in the regulation of metastasis process, including ICAM-1 (Senggunprai et al., 2013) and vascular endothelium growth factor (Zhao et al., 2012). In this study, it was found that IL-6 increased the motility of CCA cells, and the specific JAK inhibitor, AG490, significantly decreased IL-6-dependent cell migration. The results underline the role of JAK/STAT3 in the migration of CCA cells. Treatment with luteolin dramatically decreased the IL-6-mediated CCA cell migration. It should be noted that the anti-migratory effect of luteolin was detected at low concentrations which minimally inhibited cell growth. These results indicate that luteolin suppresses the metastatic potential of CCA cells, at least in part, through modulating the JAK/STAT3 signaling pathway.

In summary, for the first time, the potential anticancer activity against CCA and the underlying mechanisms of luteolin were investigated in this study. Luteolin exhibits effective cytotoxicity against CCA cells in vitro. Cell cycle arrest and apoptosis induction could be attributed in part to its proliferating inhibition. In addition, luteolin may serve as a potential important anticancer agent for CCA progression by blocking the JAK/STAT3 signaling pathway. Further investigations in vivo are needed before

luteolin can become a new option in the treatment of CCA.

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