Luteolin inhibits insulin-like growth factor 1 receptor signaling in prostate cancer cells

**Jing Fang1,*,†, Qiong Zhou1,** Xiang-lin Shi1 and Bing-hua Jiang1,2

1The Institute for Nutritional Sciences, Shanghai Institute for Biological Sciences, Chinese Academy of Sciences, Graduate School of Chinese Academy of Sciences, Shanghai 200031, China and 2The Department of Sciences, Chinese Academy of Sciences, Graduate School of Chinese Advance Access publication October 25, 2006 doi:10.1093/carcin/bgl189

Carcinogenesis vol.28 no.3 pp.713–723, 2007

**Abstract**

Prostate cancer is one of the most commonly diagnosed malignant tumors in Western countries. Overexpression of IGF-1R in prostate cancer is associated with tumor growth. These suggest that IGF-1R inhibitory agents may be of preventive and/or therapeutic value. With evidence accumulating for a chemopreventive role of flavonoids, the effects of luteolin, a bioactive flavonoid, on IGF-1R signaling in prostate cancer cells were examined. Luteolin inhibited insulin-like growth factor 1 (IGF-1) induced activation of IGF-1R and AKT in prostate cancer PC-3 and DU145 cells. Inhibition of AKT by luteolin resulted in decreased phosphorylation of its downstream targets, including p70S6K1, GSK-3β and FKHR/FKHL1. Luteolin also inhibited the IGF-1-induced activation of EGFR and MAPK/ERK signaling. Luteolin inhibited expression of cyclin D1 and increased expression of p21. As a result, luteolin suppressed proliferation and induced apoptosis of prostate cancer cells. Knockdown of IGF-1R by siRNA led to inhibition of proliferation of prostate cancer cells. Results of in vivo tumor growth assay indicated that luteolin inhibited PC-3 tumor growth. Immunoblotting of the extracts of tumor tissues showed that luteolin inhibited IGF-1R/AKT signaling. Our results provide a new insight into the mechanisms that luteolin is against cancer cells.

**Introduction**

Prostate cancer is one of the most commonly diagnosed malignant tumors in Western countries. The etiology of prostate cancer is unknown. It has been suggested that growth factor abnormalities may be involved in initiation and progression of this disease (1). Case-control studies have associated elevated levels of insulin-like growth factor 1 (IGF-1) to increased risk of the three most prevalent cancers in United States: prostate cancer, colorectal cancer and lung cancer (2). Thereby it has caused an enhanced interest in the role of IGF-1 in growth control and carcinogenesis. Insulin-like growth factors (IGF) are potent mitogens and survival factors for a variety of cancer including prostate cancer since they stimulate cancer cell growth and suppress apoptosis. These effects are mediated primarily through the insulin-like growth factor 1 receptor (IGF-1R), a tyrosine kinase receptor that shares substantial sequence homology with the insulin receptor (3).

The importance of the IGF system in prostate growth is underscored by the detection of every element of this system, including IGF-1R, IGF-1 and IGF-2 proteins, as well as IGF-binding proteins (IGFBP) (4–6), in normal, hyperplastic and/or neoplastic prostate cells and tissues. IGFBP binds IGF-1 or IGF-2 and blunts their proliferative effects on cells (7).

Prostate cancers have been shown repeatedly to relate serum IGF-1 levels (8). The nested case-control study within the Harvard Physician’s Health Study (9) and a population-based case-control study in Sweden (10) revealed a 7–8% elevation in serum IGF-1 levels among prostate cancer patients relative to age-matched controls.

IGF-1 may increase in vitro proliferation of prostate cancer cells, whereas antisense-mediated inhibition of IGF-1R expression suppresses in vivo tumor growth and prevents prostate cancer cell invasiveness (11). Blockade of IGF-1R has been shown to result in inhibition of prostate cancer cell proliferation in vitro and tumor growth in vivo (12). In human prostate cancer cell xenografts, progression to androgen independence in some experimental models is associated with increased expression of both IGF-1R and IGF-1 (13). In primary culture, prostatic epithelial cells proliferate on IGF-1 and insulin. IGF-1 is more potent than IGF-2 and insulin. IGF-2 achieves the same level of stimulation as IGF-1 at a 10-fold higher concentration and insulin as a 500-fold higher concentration (1).

IGF-1 binds to the IGF-1R, which is a heterotetrameric transmembrane glycoprotein comprising two α subunits and two β subunits. The β subunits express intrinsic tyrosine kinase activity, which is activated upon ligand binding to the α subunits. Tyrosine kinase activation results in autophosphorylation of the β subunits on specific tyrosine residues, which then act as docking sites for a range of signaling molecules, which then transmit downstream signals. The activated IGF-1R phosphorylates adaptor proteins, such as IRS-1, which is coupled to the major signaling pathway phosphatidylinositol 3-kinase (PI3K)-AKT signaling pathway (14). Because the IGF-1/IGF-1R signaling contributes to cancer progression, the inhibition of IGF-1R actions may potentially constitute a new approach to cancer treatment.

Luteolin, the 3′,4′,5,7-Tetrahydroxyflavone, is a common dietary flavonoid and has been found to have anti-tumor properties. Luteolin shows strong anti-proliferative activity against different human cancer cell lines including prostate cancer cells (15–20). However, the mechanism that luteolin...
inhbits cancer cell is still not well understood. Herein, we demonstrate that luteolin inhibited IGF-1/IGF-1R signaling in human prostate cancer cells. Luteolin inhibits tyrosine phosphorylation of IGF-1R and activation of AKT induced by IGF-1, which resulted in the inhibition of cell proliferation in vitro. It also inhibited prostate tumor growth in vivo. Our results provide a new insight into the mechanism that luteolin is against cancer.

Materials and methods

Chemicals and antibodies

The luteolin and IGF-1 were purchased from Sigma (St. Louis, MO, USA). Luteolin was dissolved in dimethyl sulfoxide (DMSO) and stored at –20°C. The antibodies against phospho-AKT (Ser473), AKT, phospho-p70S6K1, p70S6K1 and phospho-FKHR (Thr32)/FKHRL1 (Thr384) were from Cell Signaling (Beverly, MA, USA). The antibodies against IGF-1R (C-20), cyclin D1 (DCS-6), IGFBP3, phospho-ERK1/2 (E4), ERK2 and phospho-EGFR (Tyr1173) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody against β-actin was a product of Sigma. The antibody (4G10) against phospho-tyrosine kinase was a product of Upstate (Chagrin Falls, IL, USA). The EGFR inhibitor AG1478 and the chemical 3-[4,5-dimethylthiazol-2-y]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO, USA). Annexin-V-FITC was obtained from BD Pharmingen (San Diego, CA, USA).

Cell culture

The prostate cancer cells PC3, DU145 and LNCaP were grown in RPMI medium (Invitrogen) supplemented with 10% heat-inactivated FBS (Invitrogen), 100 U/ml penicillin and 100 µg/ml streptomycin, in 5% CO₂ incubator at 37°C.

Immunoblotting and immunoprecipitation

Isolation of cellular proteins and the immunoblotting were performed as described previously (21). For immunoprecipitation, 500 µg of cellular proteins was incubated with 1 µg of anti-IGF-1Rβ antibody at 4°C for 4 h. A total of 30 µl of protein A/G agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added and incubated at 4°C overnight. The beads were spun down and washed four times with lysis buffer under 4°C. A total of 36 µl of SDS sample buffer (2×) was added and boiled for 3 min. After a brief centrifugation at 3000 g, the supernatant was loaded to SDS–PAGE gel (8%). The phosphorylated IGF-1R was detected by using the antibody against phospho-tyrosine kinase.

DNA synthesis assay

DNA synthesis was measured by the incorporation of the BrdU into newly synthesized DNA using a Kit from Roche (Basel, Switzerland). Quiescent cells in serum-free medium were stimulated to synthesize DNA by adding IGF-1. In brief, the cells were plated in 96-well plates and incubated overnight. The cells were washed once with serum-free medium. In 24 h, the cells were pretreated with luteolin for 0.5 h. IGF-1 (40 ng/ml) was added and incubation was continued. Cells were harvested at different time intervals as indicated. Phosphorylation of IGF-1R under serum-starved condition. Serum-starved cancer cells were stimulated by luteolin (40 µM) for 0.5 h. IGF-1 (40 ng/ml) was added and the cells were incubated for 6 h. The cells were fixed, and BrdU incorporation was quantified using monoclonal antibody to BrdU conjugated to peroxidase as per the manufacture’s instruction.

Cell cycle analysis

Cells were seeded in a 100 mm dish at 40–50% confluence and incubated overnight. Cells were washed once with PBS and grown in serum-free medium. In 24 h, the medium was discarded and fresh serum-free medium containing IGF-1 (40 ng/ml) was provided with or without luteolin. The cells were incubated for 24 h. For cell cycle analysis, cells were trypsinized and washed twice with PBS. Then cells were fixed with 70% ice-cold ethanol, followed by the incubation of the freshly prepared nuclei staining buffer (0.1% Triton X-100 in PBS, 200 µg/ml RNase and 20 µg/ml propidium iodide) for 15 min at 37°C. Cell cycle histograms were generated after analysis of stained cells by fluorescence-activated cell sorting (FACS) with a Becton Dickinson FACScan. For each sample, at least 1 × 10⁶ events were recorded. Histograms generated by FACS were analyzed by ModFit Cell Cycle Analysis software (Verity, Topsham, ME, USA) to determine the percentage of cells in each phase.

![Fig. 1.](http://carcin.oxfordjournals.org/)

J.Fang et al.

714
Quantification of apoptotic cells
Due to DNA fragmentation, chromatin condensation and nucleus fragmentation, apoptotic cells have a less DNA content as compared to the G1 cells, which is called the subdiploid cells. The number of apoptotic cells was expressed as percent of subdiploid cells. Briefly, cells grown in medium containing 10% serum were treated with luteolin when reaching ~50% confluence. After treatment, the cells were harvested and stained with PI as described above. Subdiploid cells were determined by means of flow cytometry. Apoptotic cells were also determined by means of Annexin-V staining of the cells. Cells were grown in medium containing 10% serum. When cell confluence was ~50%, luteolin was added and the incubation continued. In 48 h, the cells were harvested and stained with Annexin-V-FITC as per the manufacture’s instruction. The stained cells were analyzed by flow cytometry within 1 h.

Cell proliferation assay
The cells were seeded in 24-well plate at 2.5 \( \times 10^4/\)well. The next day, the old medium was discarded and the cells were washed with PBS once. Fresh serum-free medium with or without luteolin was provided. IGF-1 was added at 40 ng/ml. The incubation continued for 24 h. After treatment the cells were harvested and cell numbers were determined under microscope.

Tunnel assay
Tumor samples were fixed in 10% buffered formalin for 24 h and processed conventionally. The paraffin-embedded tumor sections (5 \( \mu \)m thick) were heat immobilized, deparaffinized using xylene and then rehydrated in a graded series of ethanol. For tunnel assay, the In Situ Cell Death Detection kit from Roche (Indianapolis, IN, USA) was used. Tunnel staining of the tissue sections was performed as per the manufacture’s instruction.

Design of siRNA against IGF-1R
IGF-1R siRNA and inverted control duplex were designed as described (22). The sequences of IGF-1R siRNA duplex are: sense strand, 5'-CGACUAUCAGCAGCUAAAGTT-3'; antisense strand, 5'-CUUCAGCUGCUAGUCGTT-3'. It is homologous to 168–186 nt of human IGF-1R transcript. 5'-GAAGUCGACGCUAAGCTT-3' and 5'-GCUGAUAGUAGUGUCGACU-UCTT-3' are sense and antisense sequences of inverted control duplex, respectively. All sequences were submitted for BLAST search to ensure that only the IGF-1R gene was targetned by IGF-1R siRNA and that the inverted control sequences were not homologous to any known human genes. The vector pSilencer 2.0-U6 from Ambion (Austin, TX, USA) was used to construct siRNA according to the instruction of the manufacture. The construct against IGF-1R mRNA is named as si-IGF-1R and the inverted control is named as si-Con.

Transient transfection
PC-3 cells at 60–70% confluence were transfected with siRNA plasmids using the Cell Line Nucleofector Kit V (Cat No. VCA-1003, for PC-3 cells) from Amaxa (Gaithersburg, MD, USA) as per the manufacture’s instruction.

In vivo tumor growth assay
Male nude mice [BALB/c-A (nu/nu)] (4-week-old) were obtained from Shanghai Experimental Animal Center (Chinese Academy of Sciences, China) and maintained in pathogen-free conditions. Mouse tumorigenesis
Fig. 4. Luteolin inhibited IGF-1 induced cell proliferation of PC-3 cells. (A) Luteolin inhibited IGF-1-induced DNA synthesis. PC-3 and DU145 cells were plated in 96-well plates (20,000 cells/well) in 0.1 ml of RPMI medium containing 10% FBS and incubated overnight. The medium was removed and the cells were washed with PBS once. The serum-free medium was provided and the incubation continued for 24 h. The old medium was replaced with fresh serum-free medium in the presence or absence of luteolin. In 30 min, IGF-1 (40 ng/ml) and BrdU (10 μM) was added and the cells were incubated for 6 h. BrdU assay was performed as described under Materials and methods. The experiment was performed three times and each time triplicate cell cultures were examined. (B) Luteolin caused cell cycle arrest at G1 phase. PC-3 and DU145 cells were seeded in 100 mm dishes at 40–50% confluence and incubated overnight. The cells were washed once with PBS and supplemented with serum-free medium. In 24 h, the old medium was discarded and fresh serum-free medium containing IGF-1 (40 ng/ml) was provided in the absence or presence of luteolin (30 μM). In 24 h, the cells were harvested for cell cycle analysis as described under Materials and methods. (C) Luteolin inhibited IGF-1-induced cell proliferation. PC-3 and DU145 cells were seeded in 24-well plate at 2.5 × 10^4/well. The next day, the old medium was discarded and the cells were washed with PBS once. Fresh serum-free medium with or without luteolin was provided. IGF-1 was added at 40 ng/ml. The incubation continued for 24 h. After treatment the cells were harvested and cell numbers were determined. The experiment was performed three times and each time triplicate cell cultures were examined. *P < 0.05 versus control; #P < 0.05 versus IGF-1 alone; ΔP < 0.05 versus control. Lut, luteolin.
Luteolin inhibits prostate cancer cells

The data represent mean ± SD from three independent experiments except where indicated. Statistical analysis was performed by student t-test at a significance level of P < 0.05.

Results

Luteolin inhibited IGF-1-induced tyrosine phosphorylation of IGF-1R
IGF-1/IGF-1R system is believed to play an important role in a few cancer cells, including prostate cancer cells (2,24,25). We first determined whether or not luteolin could inhibit the activation of IGF-1R by IGF-1. The prostate cancer cells PC-3 and DU145 were tested. As shown in Figure 1A, the phospho-IGF-1R was not detectable in serum-starved cells. Once stimulated by IGF-1, the phospho-IGF-1R levels were greatly enhanced. Pretreatment of the cells with luteolin inhibited significantly the phosphorylation of IGF-1R by IGF-1 (Figure 1A). The blockade of IGF-1R activation by luteolin resulted in suppression of IGF-1-induced activation of AKT (Figure 1B). With PC3 cells, we determined the kinetics of luteolin on phosphorylation of IGF-1R and AKT. Luteolin inhibited phosphorylation of both IGF-1R and AKT during the time tested (Figure 1C). Since AKT activation is downstream of IGF-1R, more significant inhibition of AKT was observed in 2 h (Figure 1C). The effects of luteolin alone on pIGF-1R and pAKT under serum-free condition were also detected. Luteolin did not induce pIGF-1R and pAKT under this condition (Figure 1D).

We also determined the effects of luteolin on IGF-1R expression. Luteolin did not affect IGF-1R expression of the cells even the treatment was prolonged to 2 days (data not shown). Additionally, we did not find that luteolin had significant effects on IGFBP3 expression in these cells (data not shown).

Luteolin inhibited downstream signals of AKT

Because IGF-1 activates PI3K/AKT and the activated AKT in turn activates its downstream survival signals to promote cell growth and resist apoptosis, therefore, we determined some of AKT downstream targets in PC-3 cells. p70S6K1, a downstream target of AKT, plays an important role in cell protein synthesis and cell cycle progression. We found that IGF-1-induced phosphorylation of p70S6K1 was inhibited by luteolin (Figure 2A).

The FKHR and FKHRL1 are members of Forkhead family of transcription factors that are involved in tumorigenesis (26). They mediate signaling via a pathway involving PI3K/AKT (27). Once phosphorylated by AKT, they promote cell survival and regulate cell cycle (28). As indicated in Figure 2A, IGF-1 induced phosphorylation of FKHR and FKHRL1 and this induction was abrogated by luteolin. Glycogen Synthase Kinase 3 beta (GSK-3β) is a unique serine/threonine kinase that is inactivated by phosphorylation. AKT is known to phosphorylate GSK-3β and results in inactivation of this kinase, which in turn results in elevated cyclin D1 protein levels (29,30). This mechanism has been proposed to play a role in proliferation induced through the AKT signaling pathway. IGF-1 induced phosphorylation of GSK-3β (Figure 2A). Pretreatment of cells with luteolin blocked IGF-induced phosphorylation of GSK-3β (Figure 2A).

Luteolin decreased expression of cyclin D1 and increased that of p21

A number of factors regulated by AKT have been shown to be involved in regulating proliferation and apoptosis. Cyclin proteins are involved in regulating entry into the different phases of the cell cycle, and cyclin D1 is necessary for progression through G1 (31). Cyclin D1 is regulated at a number of different levels, but one primary mechanism of regulation is through protein degradation. Phosphorylation of cyclin D1 by GSK-3β has been demonstrated to cause exportation from the nucleus to the cytoplasm and target it for degradation via ubiquination (29). We found that luteolin inhibited phosphorylation of GSK-3β. So, we next determined the effects of luteolin on expression of cyclin D1. As expected, IGF-1 induced expression of cyclin D1, which was suppressed by luteolin (Figure 2B). We also determined the effects of luteolin on expression of p21, the cyclin-dependent kinase inhibitor. Expression of p21 causes cell arrest at G1 phase and inhibits cell proliferation. As shown in Figure 2C, the basal level of p21 is very low in PC-3 cells under serum-starve condition and addition of luteolin increased expression of p21 (Figure 2C). It is known that p21 can be regulated by p53. But PC-3 cells are p53 null. Our results suggest that luteolin induces p21 expression in a p53-independent way. We also checked the above proteins in prostate cancer LNCaP cells that have wild-type p53. As shown in Figure 2D, luteolin induced expression of p21 and inhibited expression of cyclin D1 as well. And luteolin induced expression of p53 in LNCaP cells (Figure 2D).

Luteolin inhibits activation of EGFR and ERK

It is known that IGF-1 can trans-activate MAPK/ERK. We determined the effects of luteolin on activation of MAPK/ERK by IGF-1. As shown in Figure 3, addition of IGF-1 to the medium induced the phosphorylation of EKR in PC-3 cells. Pretreatment of the cells with luteolin suppressed this activation (Figure 3). To know whether the inhibition of ERK phosphorylation is through inhibition of pEGFR phosphorylation, we determined the effects of luteolin on EGFR phosphorylation by IGF-1. IGF-1 induced activation of EGFR and luteolin abrogated this induction (Figure 3). AG1478, a specific inhibitor of EGFR, blocked IGF-1-induced activation of EGFR and ERK (data not shown), suggesting that luteolin inhibit ERK signaling via inhibition of EGFR transactivation.

Luteolin inhibited IGF-1-induced cell proliferation

We first determined the effects of luteolin on IGF-1-induced DNA synthesis in PC-3 and DU145 cells. As shown in Figure 4A, IGF-1 stimulated DNA synthesis in both PC3 and DU145 cells. Addition of luteolin inhibited IGF-1-stimulated DNA synthesis in a dose-dependent manner (Figure 4A). Next, we determined the effects of luteolin on cell cycle progression of PC-3 and DU145 cells stimulated by IGF-1. After a 24 h serum-deprivation, 67.40% of PC-3 cells and 63.77% of DU145 cells were in G0/G1 phase, respectively.
Stimulation of the cells with IGF-1 for 24 h decreased the ratio of cells in G₀-G₁ phase to 51.07% for PC-3 and 52.89% for DU145, respectively (Figure 4B). Treatment of the cells with luteolin in the presence of IGF-1 increased cells in G₀-G₁ phase back to 64.38% for PC3 and 65.71% for DU145, respectively. These results suggest that luteolin inhibits IGF-1-induced proliferation of prostate cancer cells and caused an arrest in G₀-G₁ phase. The effects of luteolin on IGF-1-induced cell proliferation were also determined by counting the cell numbers. As indicated, proliferation of PC-3 and DU145 cells was induced by IGF-1, which was inhibited by luteolin (Figure 4C). Proliferation of the cells treated with luteolin alone under serum-free medium was also determined. As shown in Figure 4C, luteolin inhibited PC-3 and DU145 cells proliferation under serum-starve condition.

Knockdown of IGF-1R suppressed PC-3 cell proliferation
Our above results showed that luteolin inhibited IGF-1-induced activation of IGF-1R and resulted in growth inhibitory effects on PC-3 and DU145 cells. In our subsequent experiments, we determined the role of IGF-1R on PC-3 cells proliferation by siRNA technology. As shown in Figure 5A, introducing si-IGF-1R that targets IGF-1R transcript to the cells decreased IGF-1R protein levels. However, the inverted control si-Con construct did not affect IGF-1R expression. Next, we determined the effects of IGF-1R knockdown on cell proliferation. PC-3 cells were grown in medium containing 10% serum. Knockdown of IGF-1R suppressed cell proliferation significantly (Figure 5B), indicating that IGF-1R plays an important role in proliferation of PC-3 cells. These results also suggest that IGF-1R is an important target for luteolin to inhibit prostate cancer cells proliferation. No big difference was observed between mock transfection and si-Con cells (Figure 5B). To know the importance of IGF-1R in the effects of luteolin, the inhibitory effects of luteolin on si-IGF-1R and si-Con PC3 cells were determined. As shown in Figure 5C, luteolin inhibited proliferation of both IGF-1R-knockdown and control cells to similar extent. For si-IGF-1R cells, there is no big difference at cell number between cell groups treated with solvent and cells treated with luteolin in day 1 and day 2 (Figure 5C). However, in si-Con group, the difference at cell number between solvent-treated cells and luteolin-treated cells is quite big during whole process (Figure 5C). These data suggest an important role of IGF-1R in the effects luteolin. In day 3, number of luteolin-treated cells of both groups decreased due to cell death. Proliferation of IGF-1R-knockdown cells was still inhibited by luteolin (Figure 5C), suggesting that luteolin may have other targets beside IGF-1R. It was reported that luteolin induced apoptosis of cancer cells via death receptor 5 (32) and fatty acid synthase (33).

Luteolin induced apoptosis of PC-3 cells
We determined whether luteolin could induce apoptosis of prostate cancer cells. PC-3 cells were tested. The experiments performed under serum-containing medium. Before we proceeded, we first determined the effects of luteolin on serum-induced activation of IGF-1R. As shown in Figure 6A, luteolin inhibited serum-stimulated phosphorylation of IGF-1R dramatically. The apoptosis of cells was analyzed by means of determination of subdiploid of cells and of Annexin-V staining. As shown in Figure 6B and C, both determinations showed that luteolin induced apoptosis of PC-3 cells.

Luteolin inhibits prostate tumor growth in nude mice
Finally, we determined the effects of luteolin on PC-3 xenograft in nude mice. The in vivo study was conducted with a s.c. xenograft model using PC-3 cells. As indicated in Figure 7A, growth of PC-3 tumors was inhibited significantly...
by administration of nude mice with luteolin. There was no evidence of systemic toxicity to the mice as evidenced by normal food intake and body weight (data not shown). Tunnel assay indicates there are more apoptotic cells in tumors treated with luteolin than those in tumors treated with solvent (Figure 7B). Western blot of extracts of tumor tissues showed that IGF-1R/AKT signaling was inhibited by luteolin (Figure 7C). Likewise, the expression of cyclin D1 and PCNA were also inhibited by luteolin (Figure 7C). Inhibition of PCNA expression indicates that luteolin suppressed tumor cell proliferation. We also determined the effects of luteolin on PC-3 tumor growth at different doses. As shown in Figure 7D, luteolin at 5 mg/kg suppressed the PC-3 tumor growth, which is consistent with the results of Figure 7A.

Discussion

The prostate cancer ranks as the most common malignancy and the second leading cause of cancer-related deaths in American males (34). The limited available options for the treatment of prostate cancer have prompted the need for
Fig. 7. Luteolin inhibited growth of PC-3 xenograft in nude mice. (A) Luteolin inhibits growth of PC-3 xenograft in nude mice. Twenty mice were randomly divided into two groups (n = 10 mice/each group). The mice of the treatment group were administered I.P. with luteolin at 5 mg/kg. The mice of control group received solvent at equal volume. Treatment was begun 3 days after implantation and continued daily throughout the study. *P < 0.05 versus control. For details, please see Materials and methods. (B) Tunnel assay of the tissues. (C) Immunoprecipitation and immunoblot of tissue extracts. After harvest, the PC-3 tumors were snap-frozen in liquid nitrogen and crushed rapidly. Cold RIPA lysis buffer was added and the samples were transferred to 1.5 ml tube and incubated on ice for 0.5 h. Specimens were centrifuged and the supernatant was collected for immunoblotting. Tyrosine phosphorylation of IGF-1R was performed as described under Materials and methods. (D) Dose-dependent study of luteolin on PC-3 tumor growth. The mice of the treatment groups were administered I.P. daily with luteolin at 5 and 10 mg/kg, respectively. The mice of control group received solvent at equal volume. (a) Representative mice receiving solvent alone and luteolin. (b) Tumor size of each group. (c) After treatment, the tumors were harvested and weighed. The data represent mean ± SD (n = 10).
developing alternative strategies for the management of prostate cancer. Based on the accumulating evidence, investigations are being pursued to modulate the IGF system as a possible means of cancer prevention or treatment (11,35,36). To reduce the risk of prostate cancer that is initiated by IGF systems, three strategies may be applicable (i) inhibiting expression of IGF and/or IGF receptor, (ii) increasing the interaction between IGF and IGFBP and (iii) blocking the interaction between IGF and IGF receptor. Chemoprevention by the use of dietary or nontoxic synthetic agents has offered a viable option to block neoplastic inception or delay disease progression. Because prostate cancer is typically diagnosed in men ages 50 years and older, even a slight delay in the onset and subsequent progression of this disease with dietary agents could have important health benefits (37). The dietary polyphenols have been suggested for prevention of many cancers including prostate cancer (37–43). We demonstrated here that luteolin inhibits prostate cancer cells through IGF-1/IGF-1R system. IGF-1 binds to IGF-1R and induces receptor tyrosine phosphorylation and thereafter activation of intracellular PI3K/AKT signal pathway. In this work, we found that luteolin blocked IGF-1-induced activation of IGF-1R and AKT. AKT is the major signal for cell survival and proliferation. Both PC-3 and DU145 cells have high levels of IGF-1R and are sensitive to IGF-1 stimulation (Figure 1). Luteolin inhibited IGF-1-induced phosphorylation of AKT and its downstream targets, such as p70S6K1, GSK-3β and FKHR. Luteolin inhibited expression of cyclin D1 (Figure 2B) and increased expression of p21 (Figure 2C) of PC-3 cells. PC-3 cells are p53 null. So, regulation of p21 by luteolin is through p53-independent pathway, which is consistent to Kobayashi’s reports (44). Regulation of p21 can be p53-dependent and -independent (45) and MDM2 functions as a negative regulator of p21 independent of p53 (46). Activation of AKT induces phosphorylation of MDM2 and this facilitates nucleus entry of MDM2 and inactivation of AKT results in suppression of entry of MDM2 into the nucleus (47). So, luteolin may up-regulate p21 expression through IGF-1R/AKT/MDM2 pathway. Inactivation of p70S6K1, inhibition cyclin D1, or induction of p21 results in cell cycle arrest and cell proliferation inhibition (31,48,49).

Among the subgroups of MAPKs, the ERKs are key mediators of the mitogenic potential of growth factors. The mitogenic effects of IGF-1 and EGF in prostate cancer cells converge at the level of the MAPK/ERK (50). It has been reported that the interruption of EGFR by anti-EGFR antibody result in a loss of IGF-1 responsiveness (51). Our results show that IGF-1 is a potent activator of MAPK/ERK in prostate cancer cells (Figure 3). Treatment of the cells with luteolin blocked the activation of ERK and EGF by IGF-1 (Figure 3). We found that activation of ERK by IGF-1 is through phosphorylation of EGFR, because the EGFR inhibitor AG1478 abrogated the activation of ERK (data not shown). The results suggest that the IGF-1R/EGFR/ERK pathway is also involved in luteolin inhibited cell proliferation by IGF-1.

With regard to IGFs-mediated activation of their receptors, recent reports indicate that bioactive forms of IGFs are tightly controlled by the presence of IGFBPs. Recent studies demonstrated that higher circulating IGF-1 level and/or lower IGFBP3 level are strongly and positively correlated with increased risk of prostate cancer. IGFBP3 is one of the important members of IGFBP family, having a high affinity for IGFs that exists in ternary complex with IGF and an acid-labile unit (2,52). We did not found that luteolin had significant effects on expression of IGFBP3 in vitro.

Inhibition of IGF-1R by luteolin resulted in suppression of proliferation of PC-3 and DU145 cells (Figure 4). To address the importance and specificity of luteolin’s inhibitory effects on IGF-1R of prostate cancer cells, we knocked down IGF-1R by siRNA and determined cell proliferation. Inhibition of IGF-1R expression decreased significantly PC-3 cell proliferation (Figure 5B) and the si-IGF-1R cells were more sensitive to luteolin than were the si-Con cells (Figure 5C), indicating the IGF-1R plays an important role in the effect of luteolin on PCI-3 proliferation.

At last, we tested the effects of luteolin on prostate cancer growth by in vivo experiments. Luteolin inhibited significantly PC-3 xenografts growth in nude mice (Figure 7A). Immunoblot of the extract of tumors (Figure 7C) and tunnel assay of the tumor tissues (Figure 7B) indicated that administration of luteolin inhibited IGF-1 signaling, suppressed cell proliferation and induced apoptosis of PC-3 cells. The effects of luteolin at different doses on tumor growth were also determined. Both treatments at 5 and 10 mg/kg (luteolin/mice) inhibited significantly the tumor growth (Figure 7D). However, no significant difference was observed between 5 and 10 mg/kg groups. This may be due to, (i) 5 mg/kg is a sufficient dose, (ii) the treatment time is not long enough or (iii) the sample size is not big enough. This needs further investigation in our future work.

IGF-1R is overexpressed in many human carcinomas, such as prostate, breast and colon cancers (2,24,25). Blockade of IGF-1R has been convincingly shown to inhibit tumorigenesis and block tumor invasion and metastasis. A variety of approaches aimed at targeting IGF-1R has been or are being developed for potential anticancer therapies. Targeting of IGF-1R to block its signaling can be obtained by interference with ligand receptor interactions, receptor synthesis and expression, receptor tyrosine kinase activity, or combinations of these strategies. A direct strategy to inhibit IGF-1R activity is to inhibit its tyrosine kinase by small-molecular inhibitors. The major advantage of this approach is that small molecules have a considerable higher bio-availability compared to antibodies, dominant-negative receptors and antisense oligonucleotides. Luteolin is a nontoxic dietary flavonoid and is bio-available easily. Our results suggest that luteolin is a potent inhibitor of IGF-1/IGF-1R system and may be beneficial in preventative and/or therapeutic treatment of prostate cancer.

Acknowledgements
This work was supported by grants from Science and Technology Commission of Shanghai Municipality (No. 05DJ14009 and 04DZ14007) and a grant from National Natural Science Foundation of China (No. 30470361 and 30570962).

Conflict of Interest Statement: None declared.

References

Received March 29, 2006; revised September 27, 2006; accepted September 29, 2006