

Original Paper

Formononetin Promotes Cell Cycle Arrest via Downregulation of Akt/Cyclin D1/CDK4 in Human Prostate Cancer Cells

Tianyu Li^a Xinge Zhao^b Zengnan Mo^a Weihua Huang^c Haibiao Yan^a Zhian Ling^c Yu Ye^c

^aInstitute of Urology and Nephrology, First Affiliated Hospital of Guangxi Medical University, Nanning,

^bSchool of Basic Medical Sciences, Guilin Medical University, Guilin, ^cDepartment of Emergency, Western Hospital, First Affiliated Hospital of Guangxi Medical University, Nanning, China

Key Words

Formononetin • CDK4 • Cyclin D1 • Akt • Prostate cancer

Abstract

Background: Formononetin is an O-methylated isoflavone isolated from the root of *Astragalus membranaceus*. It has already been reported that formononetin could inhibit cell proliferation and induce cell apoptosis in several cancers, including prostate cancer. This study aimed to further investigate whether cell cycle arrest is involved in formononetin-mediated antitumor effect in human prostate cancer cells, along with the underlying molecular mechanism.

Methods: Human prostate cancer cells PC-3 and DU145 were respectively treated with various concentrations of formononetin. The inhibitory effect of formononetin on proliferation of prostate cancer cells was determined using MTT assays and flow cytometry. Next, formononetin-induced alterations in cyclin D1, CDK4 and Akt expression in PC-3 cells were detected by real-time PCR and western blot. **Results:** Formononetin dose-dependently inhibited prostate cancer cell proliferation via the induction of cell cycle arrest at G0/G1 phase *in vitro*, which was more evident in PC-3 cells. Meanwhile, concomitant with reduced phosphorylation of Akt in PC-3 cells, formononetin remarkably downregulated expression levels of cyclin D1 and CDK4 in a dose-dependent manner. More interestingly, in the *in vivo* studies, formononetin showed a noticeable inhibition of tumor growth in recipient mice. **Conclusion:** Formononetin could exhibit inhibitory activity against human prostate cancer cells *in vivo* and *in vitro*, which is associated with G1 cell cycle arrest by inactivation of Akt/cyclin D1/CDK4. Therefore, formononetin may be used as a candidate agent for clinical treatment of prostate cancer in the future.

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Yu Ye

Department of Emergency, Western Hospital, First Affiliated Hospital of Guangxi Medical University, 530021 (China)
Tel. +86-0771-5350000, Fax +86-0771-5350000 E-Mail gxzengnanmo@163.com

Introduction

In spite of recent advances in molecular diagnostic technologies and new drug development, prostate cancer (PCa) remains the most common cancer in men older than 50 years of age, and the second leading cause of cancer-related deaths in men in America [1-3]. Until now, the etiology of PCa has not been entirely elucidated, which may involve age, ethnicity, family history of prostate cancer, genetic susceptibility and lifestyle factors [4]. The incidence of PCa was reported to be much higher in North America than in Western Europe, Oceania, and Asia [5]. Moreover, men from Asia are likely to acquire an increased incidence of PCa after emigration to America, suggesting that diet diversity may play a major role in the initiation, promotion, and progression of PCa [6]. Considering the difference in soy food consumption between Asian and Western countries, researchers began to focus on whether soy could contribute to the lower occurrence of PCa in Asia [7].

Phytoestrogens, sometimes called dietary estrogens, are present at high concentrations in many soy products. Owing to the high structural similarity with estrogen, phytoestrogens may have either estrogenic or antiestrogenic effects. Previous studies have demonstrated that some phytoestrogens, such as genistein, could inhibit growth of PCa cells *in vitro*, indicating the antiestrogenic activity of phytoestrogens on prostate cancer [8]. Formononetin ($C_{16}H_{12}O_4$) is a traditional Chinese herbal medicine isolated from red clover, and distributed extensively in Guangxi Province of Southern China [9]. As a typical phytoestrogen, formononetin has been demonstrated to possess a wide range of biological activities including antioxidant, antiviral and cardioprotective effects [10]. Remarkably, some other studies reported formononetin-induced tumor cell apoptosis in some cancer types *in vitro* and *in vivo* [11-14]. For instance, it was shown that formononetin could induce mitochondrial apoptosis in PCa cells via downregulation of IGF-1/IGF-1R signaling pathway, implying the potential application of formononetin in antitumor therapy against prostate cancer [15]. In addition, our previous studies have confirmed the prevention of breast carcinogenesis by formononetin-triggered cell cycle arrest at G0/G1 phase [16]. Therefore, we postulated that formononetin may likewise inhibit growth of PCa through cell cycle arrest. Moreover, in order to better determine the molecular mechanism of formononetin, we here explored the roles of cyclin D1, CDK4 and Akt in prostate neoplasia.

Materials and Methods

Drugs and animals

Formononetin ($C_{16}H_{12}O_4$, purity > 98%, verified by high performance liquid chromatography) was purchased from Phytomarker Company (Tianjin, China). Formononetin was dissolved in dimethyl sulfoxide (DMSO), and then diluted with RPMI-1640 to the desired final concentration. The final concentration of DMSO in the assay was under 0.1% (v/v).

Cell culture

Human prostate cancer cell lines (PC-3, DU145) were obtained from American Type Culture Collection (Manassas, VA, USA). Cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA) in a humidified atmosphere of 5% CO₂ at 37°C.

MTT assay

Cells were seeded into 96-well plates (4×10^3 cells per well) and cultured for 24 h. After incubation with increasing concentrations of formononetin (0, 10, 20, 30, 40, 60, 80, 100 μ M) for 48 h, cells were exposed to MTT (5 mg/mL) in phosphate buffered saline (PBS). Subsequently, the medium was removed and the formed formazan was dissolved in 150 μ L dimethyl sulfoxide (DMSO, Sigma). The optical density (OD) for each well was measured at 570 nm by a microculture plate reader (Bio-Rad, USA).

Flow cytometry assay

After 48 h treatment of formononetin (0, 20, 40 and 80 μ M), PC-3 cells were harvested and fixed in ice-cold 70% ethanol at 4°C for 30 min. Then cells were washed with PBS, resuspended in 500 μ L of binding buffer, and stained with annexin V-FITC and propidium iodide (PI) for 30 min in the dark at 37°C. The Annexin V-FITC apoptosis detection kit (Boster, China) was used to determine the percentage of cells in G0/G1, S and G2/M cell-cycle phases according to the manufacturer's instruction.

Real-time PCR assay

PC-3 cells were treated with different concentrations of formononetin (0, 25, 50 and 100 μ M) for 48 h. Then total RNA was extracted from cells using the TRIzol reagent (Gibco-BRL, USA) according to the manufacturer's instruction. The RNA pellet was washed with ice-cold 75% ethanol, air-dried and dissolved into RNase-free water. cDNA was reverse-transcribed from total RNA following the RevertAid First Strand cDNA Synthesis Kit (Fermentas, USA). Amplification was conducted with an initial 10 min step at 95°C, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and then 72°C for 45 s. PCR products were separated on 1% agarose gel stained with ethidium bromide. And data were analyzed by a comparative threshold (CT) cycle method (Bulletin, PE Applied Biosystems). β -actin was used as an internal control.

Western blot assay

PC-3 cells were incubated with various concentrations of formononetin (0, 20, 40 and 80 μ M) for 48 h. Cell extracts were prepared in ice-cold lysis buffer. Then equivalent amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA). After blocking with TBST (Tris-buffered solution, 0.05% Tween 20, pH 7.6) containing 5% nonfat milk for 2 h, the membranes were immunoblotted with various primary antibodies as indicated: Anti-CDK4 (1:1000), anti-cyclin D1 (1:1000), anti-Akt (1:1000), anti-p-Akt (1:1000) and anti- β -actin (1:1000). The membrane was visualized using electrochemiluminescence (ECL) Western blot detection reagents (Beyotime, China). Image pro plus 5.02 software (Media Cybernetics, Bethesda, MD, USA) was used to quantify each band intensity. The intensity of each band was normalized to the corresponding intensity of β -actin or Akt, which was used as a loading control.

Mice and tumor xenografts

Nude mice were used for xenograft studies with subcutaneous injection of PC-3 cells (1×10^7 , 0.2 ml per nude mouse). When the tumor grew to 2.0 \times 2.0 cm size, it was cut into little pieces (1 mm \times 1 mm \times 1 mm in size), and implanted into another 40 new recipient nude mice. After about 10 days, recipient mice were randomly divided into four groups (n=10). Among them, formononetin groups respectively received intraperitoneal administration of formononetin (15, 30 and 60 mg/kg/d) for 20 days, while the control group received PBS injections. During the 20-day period, the size of tumors was regularly recorded. Then the recipient mice were killed and tumors were excised. The tumor inhibitory rate was calculated by the following formula:

$$\text{The tumor inhibitory rate (\%)} = 1 - (W_{\text{treated}} / W_{\text{control}}) \times 100\%$$

Statistical analysis

All statistical data were expressed as mean \pm standard deviation. Statistical analysis was conducted by one-way ANOVA and Student's t-test using the Statistical Package for Social Sciences (SPSS) 13.0 software. A probability (p)-value < 0.05 was used as the criterion of statistical significance.

Results*Inhibited proliferation in both PC-3 and DU145 cells by formononetin*

To study the inhibitory effect of formononetin on prostate cancer cells, PC-3 and DU145 cells were respectively treated with various doses of formononetin for 48 h. According to MTT assay, PC-3 cells were more sensitive to formononetin than DU145 cells. As shown in

Fig. 1. Formononetin inhibited cell proliferation in both PC-3 and DU145 cells. All cells were respectively treated with various concentrations of formononetin for 48 h, and then cell viability was determined by MTT assay. 10 independent experiments were repeated. ** $p < 0.05$ vs. control group (0 μM); $n = 10$.

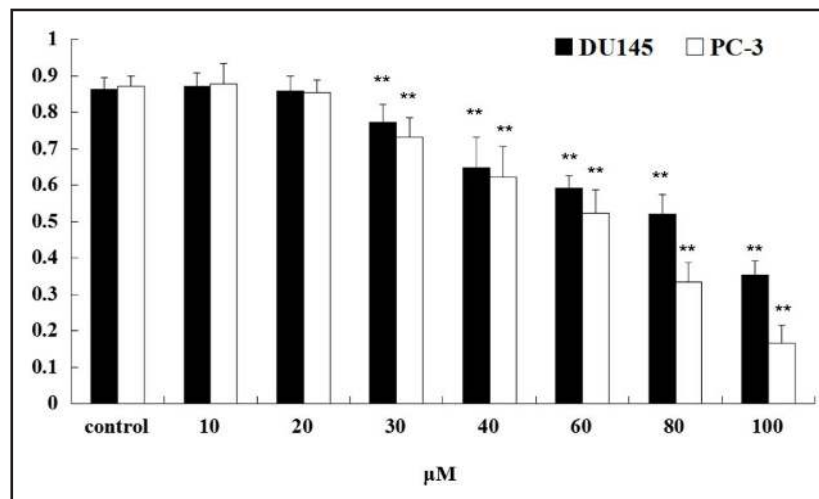


Fig. 2. Formononetin induced cell cycle arrest of PC-3 cells. Cells were treated with or not treated with formononetin (0, 20, 40 and 80 μM) for 48 h. Then Annexin V-FITC and PI staining followed by flow cytometry were used to assess cell cycle. Data were the mean \pm SD of 3 independent experiments.

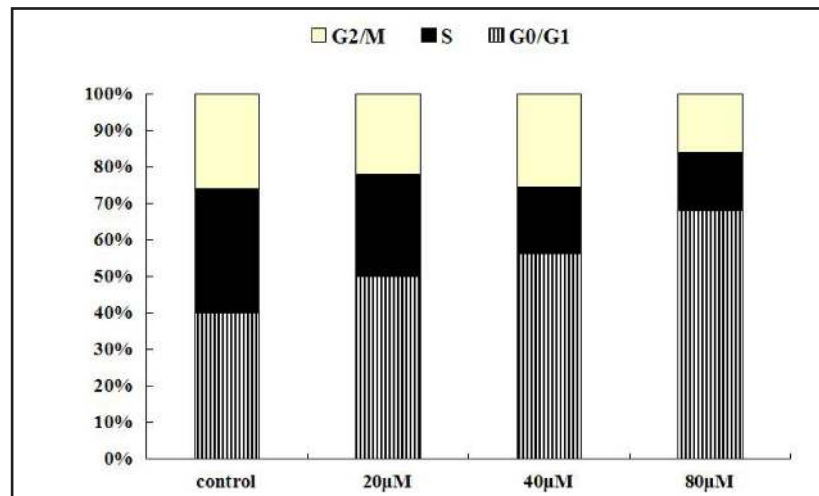


Fig. 1, formononetin inhibited the viability of PC-3 cells in a dose-dependent manner, which remarkably decreased from 0.879 ± 0.054 (0 μM) to 0.167 ± 0.048 (100 μM) ($p < 0.05$). By comparison, the viability of DU145 cells was reduced from 0.871 ± 0.038 (0 μM) to 0.354 ± 0.037 (100 μM), and obviously lower than that of PC-3 cells under the same conditions. All the above observations suggested the inhibition of formononetin on proliferation of PCa cells. And we preferred to use PC-3 cells rather than DU145 cells in our successive experiments.

Induction of cell cycle arrest in PC-3 cells by formononetin

To further elucidate how formononetin inhibits the proliferation of PC-3 cells, flow cytometry assay was performed to observe the cell DNA content for assessment of cell cycle in different groups. It was found that the percentage of cells that stayed at the G0/G1 phase was only 40% in the vehicle control group (0 μM), as shown in Fig. 2. However, formononetin-treated cells showed a gradual increase in the percentage of G0/G1 phase with concentration (50%, 62% and 68%) ($p < 0.05$), indicating that formononetin exerts inhibitory effects on PC-3 cells growth via induction of cell cycle arrest.

Downregulation of CDK4 and cyclin D1 mRNA expressions in PC-3 cells by formononetin

PC-3 cells were treated with gradient concentrations of formononetin (0, 25, 50, and 100 μM) for 48 h, and then the mRNA expressions of CDK4 and cyclin D1 were evaluated to explore the potential mechanisms of formononetin-mediated induction of G0/G1 cell cycle arrest. By real-time PCR, we found that the mRNA expression levels of CDK4 and cyclin D1

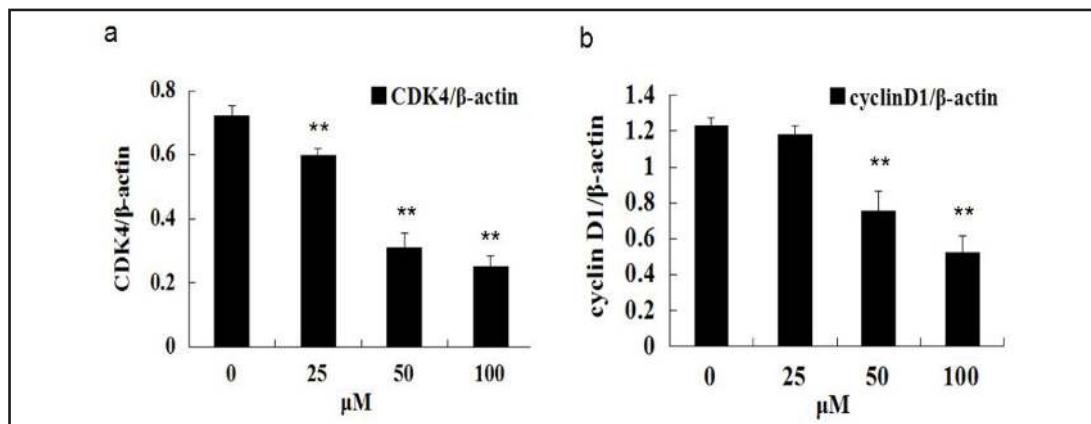
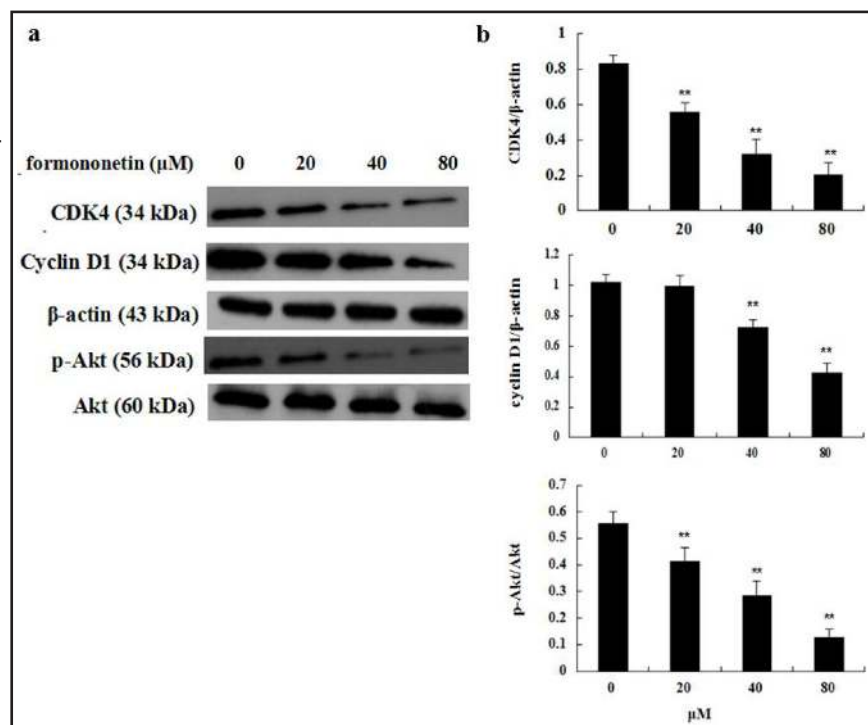


Fig. 3. Formononetin suppressed CDK4 and cyclin D1 mRNA expressions in PC-3 cells. After treatment of formononetin (0, 25, 50 and 100 μM) for 48 h, the mRNA expression levels of CDK4 and cyclin D1 were determined by real-time PCR. Data were the mean ± SD of 3 independent experiments. **p < 0.05 vs. control group (0 μM); n = 3.

Fig. 4. Formononetin reduced expression of cyclin D1 and CDK4 proteins, and phosphorylation of Akt in PC-3 cells. Cells were respectively treated with formononetin (0, 20, 40 and 80 μM) for 48 h, and then expression levels of CDK4, cyclin D1 and p-Akt were determined by Western blot (a). The quantification of band intensity relative to β-actin or Akt were presented as mean ± SD. **p < 0.05 vs. control group (0 μM); n = 3.



were obviously downregulated by formononetin in a dose-dependent manner ($p < 0.05$) (Fig. 3).

Reduced protein expressions of CDK4, cyclin D1, and Akt in PC-3 cells by formononetin

Next, western blot analysis was used to confirm the involvement of CDK4 and cyclin D1 in formononetin-induced cell cycle arrest in PC-3 cells, as well as phosphorylation levels of Akt. Consistent with the results above, formononetin (20, 40 and 80 μM) markedly and dose-dependently reduced the levels of CDK4 and cyclin D1 proteins when compared with the vehicle control group (0 μM) ($p < 0.05$), as shown in Fig. 4. Similarly, Akt was inactivated with the treatment of formononetin in a dose-dependent manner ($p < 0.05$). Overall, these data suggested that the anticancer effects of formononetin on PC-3 cells were highly concerned with the inactivation of CDK4, cyclin D1 and Akt.

Table 1. Inhibitory effects of formononetin on tumor growth (mean \pm SD, n = 10). ** p < 0.05 vs. control

Group	Dose mg/kg	N	Body Weight(g)		Tumor weight (g)	Inhibitory Rate (%)
			Pre	post		
Control	-	10	20.32 \pm 2.41	27.34 \pm 3.31	1.15 \pm 0.38	-
Formononetin	15	10	19.99 \pm 1.95	26.99 \pm 4.21	1.02 \pm 0.35	11.3
Formononetin	30	10	20.01 \pm 1.55	26.83 \pm 3.72	0.89 \pm 0.22**	22.61
Formononetin	60	10	19.63 \pm 1.46	27.03 \pm 4.23	0.63 \pm 0.35**	45.22

Suppression of tumor growth in recipient mice by formononetin

Based on the *in vitro* results, we further assessed the inhibitory effect of formononetin on prostate cancer cells *in vivo*, in which nude mice bearing tumor xenografts were treated with formononetin or not. Compared with the negative control group, tumor growth was sharply inhibited by formononetin in a dose-dependent manner (p < 0.05), demonstrating its inhibitory effect on PCa growth *in vivo* (Table 1). And the mean tumor weight in mice treated with formononetin was significantly lower than control group, especially at the dose of 60 mg/kg (0.63 \pm 0.35 vs 1.15 \pm 0.38, p < 0.05), with an inhibitory rate up to 45.22%.

Discussion

As far as we know, the genetic and epigenetic alterations are responsible for the development of prostate cancer through inducing malignant transformation of normal glandular epithelia [17]. Based on its relationship with sex hormone, both estrogen and estrogen receptor (ER) may contribute to prostate carcinogenesis [18-21]. Phytoestrogens, which are increasingly receiving attention for their estrogenic or antiestrogenic effects, play important roles in inhibiting estrogen-dependent tumors via a direct correlation to dosage. In the present study, we also proved that formononetin, a typical phytoestrogen, markedly attenuated the proliferation of PCa cells in a dose-dependent manner *in vitro*, and this inhibitory effect was more salient in PC-3 cells than DU145 cells. *In vivo*, formononetin induced a dramatic decrease in tumor weight in the treated mice, further confirming the potential application of formononetin in treating human prostate cancer. A lot of studies demonstrated that the underlying mechanism of dietary phytochemicals-mediated chemopreventive activity may involve the induction of cell cycle arrest [22, 23]. In accordance with the previous studies, increased cell cycle arrest at G0/G1 phase was found in formononetin-treated human prostate cancer cells, which suggests that the induction of cell cycle arrest may be the major mechanism for its anti-proliferation effect.

Generally speaking, alterations in the genome and protein expressions are crucial to tumor malignant phenotype and progression. Previously, our studies showed that formononetin successfully induced cell cycle arrest at the G0/G1 phase by inactivating IGF1/IGF1R-PI3K/Akt pathways in MCF-7 human breast cancer cells [16]. Thus, we here continued to focus on the molecular mechanism of cell cycle arrest in PCa cells induced by formononetin. The four phases (G1, S, G2, and M phases) of the cell cycle are sequentially transitioned in response to growth factor or oncogenic stimulation, including cyclins, the regulatory units, and cyclin-dependent kinases (CDKs), the catalytic units [24, 25]. Among them, cyclin D and E, together with CDK2, CDK4, or CDK6, play major parts in the replication of DNA and mitosis via the regulation of G0/G1 phase of the cell cycle [26]. And overactivation of cyclins and CDKs can provide cancer cells a selective growth advantage [27]. Cyclin D1 is considered as a mitogenic sensor that relays and amplifies extracellular growth signals [28]. By activating its partner kinases, CDK4, elevated cyclin D1 is known to shorten the cell-cycle length, directly contributing to G0/G1 cell cycle arrest and the proliferation of many human cancer cells such as cancers of the breast [29, 30]. Accordingly, targeting cyclin D1/CDK4 complexes may be a potential way to treat prostate cancer. As expected, we here found that formononetin significantly block the expression of cyclin D1 and CDK4 (reduction in mRNA

and protein) in a dose-dependent manner in PC-3 cells, in concordance with increased G0/G1 cell cycle arrest.

PI3K/Akt signaling pathway is an important intracellular signal transduction pathway, participating in regulation of cell apoptosis and proliferation. Akt, a promoter of cell proliferation and survival, is found to be overexpressed in the tumor formation, implying that inactivation of Akt may prevent the development and progression of cancers [31]. Previous studies suggest that the activation of Akt could impair phosphorylation of glycogen synthase kinase (GSK) 3 β , followed by increased expression of cyclin D1, facilitating the G1/S progression of cell cycle [32]. In the present study, inactivation of Akt in PC-3 cells was demonstrated with treatment of formononetin, which is consistent with formononetin-triggered degradation of cyclin D1 and CDK4. Thereby, we conclude that formononetin arrests cell cycle progression at G0/G1 phase by downregulating the phosphorylation state of Akt, and subsequently decreasing the levels of cyclin D1 and CDK4 expression.

Taken together, these findings indicate that formononetin exhibits anti-tumor effects on human prostate cancer cells by leading to G0/G1 cell cycle arrest, which is mediated by inhibiting Akt-mediated signaling pathways and blocking the expressions of cyclin D1/CDK4 complexes. Considering the wide application of red clover plants in clinical, formononetin may be a promising antiproliferative agent for the treatment of prostate cancer.

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