Published in final edited form as:

Cancer Lett. 2013 September 1; 337(2): 177–183. doi:10.1016/j.canlet.2013.05.014.

Molecular interplay between cdk4 and p21 dictates G₀/G₁ cell cycle arrest in prostate cancer cells

Thippeswamy Gulappa, Ramadevi Subramani Reddy, Suman Suman, Alice M. Nyakeriga, and Chendil Damodaran*

Department of Biomedical Sciences, Paul L. Foster School of Medicine, Texas Tech University Health Sciences Center, El Paso, TX 79905, USA

Abstract

This study examined the effect of 3, 9-dihydroxy-2-prenylcoumestan (pso), a furanocoumarin, on PC-3 and C4-2B castration-resistant prostate cancer (CRPC) cell lines. Pso caused significant G₀/G₁ cell cycle arrest and inhibition of cell growth. Molecular analysis of cyclin (D1, D2, D3, and E), cyclin-dependent kinase (cdk) (cdks 2, 4, and 6), and cdk inhibitor (p21 and p27) expression suggested transcriptional regulation of the cdk inhibitors and more significant downregulation of cdk4 than of cyclins or other cdks. Overexpression of cdk4, or silencing of p21 or p27, overcame pso-induced G₀/G₁ arrest, suggesting that G₀/G₁ cell cycle arrest is a potential mechanism of growth inhibition in CRPC cells.

Keywords

Cell cycle arrest; Castration-resistant prostate cancer; Cyclins; Cyclin-dependent kinase; Growth inhibition

1. Introduction

Prostate cancer (CaP), the most common cancer in American males and the second leading cause of death in men, continues to be a major problem. According to the American Cancer Society, more than 241,000 men will be diagnosed with CaP in the United States and more than 28,000 will die from this disease this year [35]. Because prostate tumor growth is initially androgen-dependent, treatment involves hormone deprivation/ablation therapy, which entails removal of circulating androgens and opposition of androgen action with anti-androgens. This approach is extremely effective initially, but many patients relapse to castration-resistant CaP (CRPC). Other conventional therapeutic procedures, including cryotherapy, radical prostatectomy, and radiation therapy, are associated with long-term side effects, including urinary and bowel problems [28]. At present, no effective therapy is available for the treatment of CRPC [2]; hence, there is a pressing need to identify alternative chemopreventive and chemotherapeutic strategies.

In recent years, significant efforts have been made to identify novel molecular targets at various stages of clinical development of CaP and to determine whether these can be exploited for prevention and treatment of CaP [2,13,18,34]. Currently, several inhibitors

*Corresponding author. Address: Center of Excellence in Cancer Research, Department of Biomedical Sciences, Paul L. Foster School of Medicine, Texas Tech University Health Sciences Center, El Paso, TX 79905, USA. Tel.: +1 915 215 4228; fax: +1 915 783 5222. C.damodaran@ttuhsc.edu (C. Damodaran).

Conflicts of Interest

The authors declare no conflicts of interest.

specifically targeting signaling pathways, such as those involving the androgen receptor (AR) (AR antagonists and inhibitors of androgen synthesis), non-receptor tyrosine kinase (Src), phosphoinositide 3-kinase/Akt/mammalian target of rapamycin, vascular endothelial growth factor, insulin-like growth factor-1 receptor, and angiogenesis, are in different phases of clinical development (reviewed in [1]). Similarly, several small-molecule inhibitors targeting the cell cycle have been identified and are in phase I, II, and III clinical trials (reviewed in [10,32]).

Cell cycle progression is regulated by coordinated activation of cyclin-dependent kinase (cdk)/cyclin complexes (reviewed in [6]). Cyclins bind and activate specific cdks. cdk4/6, in association with cyclin D1, and cdk2, in association with cyclins E and A, phosphorylate proteins of the retinoblastoma tumor suppressor (Rb) family (reviewed in [15,41]). Phosphorylation of Rb determines whether a cell will enter S phase by release of a family of transcriptional regulators collectively called E2Fs, which are normally bound to hypophosphorylated Rb (reviewed in [37]). In addition, cdk inhibitors, such as p21 and p27, negatively regulate cell cycle progression by inhibiting the activity of the cyclin D1/cdk4/6 and cyclin E/cdk2 complexes, thereby decreasing the level of hyper-phosphorylated Rb [11].

Recent epidemiological and experimental studies have shown promise for dietary phytochemicals in chemoprevention of CaP through cell cycle inhibition [19,20,24]. Some of these agents are in different phases of clinical trials, and it may be expected that the number will increase in the future. Coumarins, a group of dietary phytochemicals, have recently attracted much attention because of their broad spectrum of pharmacological activities, including anticancer activity [3,23,31]. 3, 9-dihydroxy-2-prenylcoumestan (psoralidin, pso), a furanocoumarin, is one of the major furanocoumarins isolated from the traditional Asian medicinal plant *Psoralea corylifolia*. Pso exhibits a variety of therapeutic properties, including anticoagulant, cytotoxic, antioxidative, antimicrobial, anti-inflammatory, and anti-allergic activity [42–44]. Recent studies have provided experimental evidence documenting the anticancer properties of pso. Results of our previous studies suggest that pso induces apoptosis in CaP cells [25,38,46].

We investigated whether pso targets the cell cycle machinery that causes inhibition of growth of CRPC cell lines. Here, we report for the first time that pso induces G₀/G₁ cell cycle arrest through inhibition of cyclin/cdk complexes and induction of p21 and p27. This mechanism may be responsible for growth inhibition in CRPC cell lines.

2. Materials and methods

2.1. Reagents

Pso, anti- β -actin antibody, and propidium iodide were purchased from Sigma (St. Louis, MO). Antibodies against cdks 2, 4, and 6; cyclins D1, D2, and D3; p21 and p27; and horseradish peroxidase-conjugated anti-mouse, anti-goat, or anti-rabbit secondary antibodies; scrambled siRNA, and p21 and p27 siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against Rb, pRb, and E2F1 were obtained from Cell Signaling Technology (Beverly, MA). Alexa Fluor 488, phalloidin, and Prolong gold antifade with DAPI mountant were purchased from Invitrogen (Grand Island, NY). pCMV-cdk4 expression plasmid was purchased from OriGene Technologies (Rockville, MD).

2.2. Cell culture

Human prostate cancer cell lines PC-3 and C4-2B were grown in Dulbecco's modified Eagle medium and RPMI-1640, respectively, supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% antibiotics, in multiwell plates, at 37 °C in a humidified atmosphere of 5% CO₂. Blood samples were obtained from healthy volunteers and Human peripheral blood

mononuclear cells (PBMCs) were isolated by Ficoll-Paque centrifugation and PBMC's were seeded in six-well plates in serum-free RPMI-1640 and allowed to adhere for 3 h. Non-adherent cells were removed and adherent cells were cultured in the medium containing 10% FBS overnight. Pso stock solution (30 mM) was prepared in dimethyl sulfoxide (DMSO) and stored at -20°C until use.

2.3. Cytotoxicity assay

PC-3 and C4-2B cells and PBMCs were plated at a density of 3×10^5 cells/well in six-well plates in medium containing 10% FBS, cultured for 24 h, and treated by addition of different concentrations of pso to the medium. The control cells were treated with the same volumes of DMSO alone, which never exceeded 0.002% of the total volume of the medium. After each treatment, cells were incubated at 37°C for 24 h in an atmosphere of 5% CO_2 . Viable cells were counted by Trypan blue exclusion using a hemocytometer. Results were expressed as a percentage of the number of cells in DMSO-treated control cultures, and the IC_{50} values were calculated using non-linear regression analysis (percent survival versus concentration).

2.4. Cell cycle analysis

Cells were plated at a density of 3×10^5 cells/well in six-well plates. After overnight attachment, cells were treated with pso (25 μM for PC-3 and 30 μM for C4-2B) for the times indicated in the figures. Cells were stained with 0.5 g/L propidium iodide and subjected to cytometric analysis using LSR II (Becton Dickinson, Franklin Lakes, NJ) as described previously [27].

2.5. Protein extraction and western blotting

After pso treatment, cells were washed with ice-cold PBS and lysed in Pierce M-PER (Thermo Scientific, Waltham, MA) lysis buffer containing Pierce 1 $\times$ Halt protease inhibitor cocktail. Proteins in cell lysates (25 μg protein) were resolved on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gels and electrotransferred to nitrocellulose membranes. Membranes were blocked with 5% non-fat milk in Tris-buffered saline containing 0.5% Tween 20 for 1 h, incubated with primary antibody overnight at 4°C , followed by incubation with peroxidase-conjugated secondary antibody, and visualized using an enhanced chemiluminescence detection system.

2.6. Overexpression of cdk4

Cells in exponential growth phase were plated 12–16 h before transfection at a density of 5×10^5 cells/well in six-well plates. Cells were transfected with either pCMV backbone vector or pCMV-cdk4 expression plasmid using TransIT-2020 Transfection Reagent (Mirus Bio, Madison, WI) according to the manufacturer's protocol.

2.7. siRNA transfection

Cells were plated, grown to 60–70% confluence, and transfected with 10 nM p21 or p27siRNA or control siRNA for 48 h using TransIT-siQUEST[®] Transfection Reagent (Mirus Bio) according to the manufacturer's protocol. Cells were treated with pso for 24 h in fresh medium. Protein expression levels were assessed by western blotting.

2.8. Immunofluorescence staining

Cells were plated at a density of $3\text{--}4 \times 10^5$ cells/well on glass coverslips in six-well plates and allowed to attach and grow to 60% confluence overnight. Following treatment with pso for 24 h, cells were washed three times with PBS, fixed in 4% paraformaldehyde for 20 min at room temperature, and permeabilized with 0.2% Triton X-100 for 20 min. To detect p21

and p27 expression, cells were incubated with mouse anti-p21 or anti-p27 antibody overnight at 4 °C, followed by anti-mouse secondary antibody conjugated to Alexa Fluor 488 for 2 h at room temperature. Finally, cells were incubated with rhodamine–phalloidin for 15 min to stain F-actin. Coverslips were washed extensively with PBS, mounted using Antifade with DAPI mountant, and analyzed using a Leica laser scanning confocal microscope.

2.9. Quantitative real-time reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from cells using the RNeasy Micro Kit (QIAGEN, Valencia, CA) and cDNA was synthesized using the Applied Biosystems cDNA synthesis kit. Quantitative real-time PCR was performed using the $2^{-\Delta\Delta CT}$ method and SYBR Green super-mix (QIAGEN, Hilden, Germany) on the Applied Biosystems multicolor real-time PCR detection system. Cycle threshold values were normalized to amplification measured for β -actin. Primers used for human p21 were (forward) 5'-CGATGCCAACCTCCTCAACGA-3' and (reverse) 5'-TCGCAGACCTCCAGCATCCA-3' and, for p27, (forward) 5'-TGCAACCGACGATTCTTCTACTCAA-3' and (reverse) 5'-CAAGCAGTGATGTATCTGATAACAAGGA-3. Amplification was performed under conditions of 95 °C for 10 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s.

2.10. Statistical analysis

GraphPad Prism 5 software (La Jolla, CA) was used for all statistical analysis. Student's *t* test was employed to assess the statistical significance of differences between control and pso-treated groups. $P < 0.05$ was considered statistically significant. Western blot bands were scanned and quantified using ImageQuant-TL v2005 software (GE Healthcare, Chalfont St. Giles, UK). Protein levels were normalized to that of β -actin and expressed as fold change.

3. Results

3.1. Pso inhibits cell viability and induces G₀/G₁ cell cycle arrest in PC-3 and C4-2B cell lines

CRPC cell lines were plated and treated with different concentrations (5–30 μ M) of pso, and cell viability was measured by Trypan blue exclusion assay. A concentration-dependent decrease in cell viability was observed in both PC-3 and C4-2B cells after 24 h of pso treatment ($P < 0.05$; Fig. 1A). In contrast, pso treatment did not exhibit significant toxicity toward human PBMCs up to a concentration of 30 μ M (Fig. 1B). To identify the mechanism underlying pso-mediated growth inhibition in CRPC cell lines, we treated exponentially growing PC-3 and C4-2B cells with 25 μ M and 30 μ M pso, respectively, for 12, 24, and 48 h. Pso treatment caused significant G₀/G₁ arrest in both cell lines. In PC-3 cells, the cells in G₀/G₁ phase accounted for 67% of the total population at 24 h and 71% at 48 h, whereas 51% of vehicle-treated cells were in G₀/G₁ arrest at both time points (Fig. 1C). Similarly, C4-2B cells in G₀/G₁ accounted for 71% at 24 h and remained at the same level up to 48 h, whereas 49% of vehicle-treated cells were in G₀/G₁ arrest at both time points (Fig. 1D). The accumulation of cells in G₀/G₁ was accompanied by a corresponding decrease in the percentage of cells in the S and G₂/M phases of the cell cycle. These results suggest that pso causes growth arrest in CRPC cell lines, possibly due to G₀/G₁ cell cycle arrest.

3.2. Mechanism underlying pso-induced G₀/G₁ cell cycle arrest in CRPC cell lines

Cell cycle progression in eukaryotes involves the coordinated and sequential activation of cdks in association with the corresponding cyclins. In general, the G₀/G₁ to S phase

transition is regulated by complexes of cyclins and cdk family proteins [14,15]. Having confirmed that pso causes significant G₀/G₁ cell cycle arrest in CRPC cell lines, we wished to determine the underlying molecular mechanism. First, we investigated the expression patterns of cyclins and cdks responsible for G₀/G₁ cell cycle regulation by immunoblotting. In PC-3 cells, pso treatment caused a marked decrease in the expression of cyclins D1, D2, and D3, whereas, in vehicle-treated cells, upregulation of these three markers was seen in a time-dependent (at 12 and 24 h) manner (Fig. 2A). In contrast, in C4-2B cells, pso caused a moderate decrease in expression of cyclins D1 and D2, compared to vehicle-treated cells (Fig. 2A).

In addition, induction of cdk2, cdk4, and cdk6 expression was evident in vehicle-treated PC-3 and C4-2B cells, whereas these proteins were downregulated in pso-treated cells at 12 and 24 h (Fig. 2B). More precisely, pso-induced p21 was expressed at a higher level in PC-3 cells than in pso-treated C4-2B cells (Fig. 2C), whereas pso-induced p27 was expressed at a higher level in C4-2B cells than in PC-3 cells (Fig. 2C). Taken together, these results strongly suggest that cdk4, p21, and p27 may play important roles in pso-induced G₀/G₁ cell cycle arrest in CRPC cell lines.

3.3. Pso treatment caused inhibition of Rb phosphorylation and E2F1 expression in CRPC cell lines

The active, phosphorylated form of Rb is also believed to play a critical role in the regulation of cell cycle progression at the G₀/G₁ to S phase transition [4]. We, therefore, examined whether pso disrupts the level of Rb phosphorylation in CRPC cell lines. The results shown in Fig. 2D demonstrate that pso treatment led to a time-dependent reduction in phosphorylated Rb (pRB) in both PC-3 and C4-2B cells. Transcription factor E2F family members phosphorylate Rb, which releases free E2F and reduces the levels of cell cycle regulators, resulting in inhibition of cell growth [36]. Hence, we analyzed the effect of pso on E2F1 protein levels. Pso treatment resulted in a strong, time-dependent decrease in E2F1 levels in both PC-3 and C4-2B cells, compared to control cells (Fig. 2E).

3.4. Transcriptional regulation and nuclear localization of p21 and p27 in pso-treated CRPC cell lines

We observed elevated expression of p21 and p27 upon pso treatment. To gain further insight into the roles of these proteins in pso-induced G₀/G₁ arrest, we examined the effect of pso on p21 and p27 transcription in PC-3 and C4-2B cells by real-time RT-PCR. Pso treatment resulted in 6-fold and 5-fold increases in p21 mRNA at 24 h in PC-3 and C4-2B cells, respectively (Fig. 3A and B). However, pso caused a moderate increase (1.9-fold and 1.85-fold) in p27 mRNA in PC-3 and C4-2B cells, respectively (Fig. 3C and D).

It is well established that nuclear accumulation of p21 and p27 inhibits cell cycle progression [9,26]. Here, we sought to determine whether pso-induced G₀/G₁ arrest might be involved in changes in p21 and p27 subcellular localization. We carried out immunofluorescence staining and confocal microscopic analysis of pso-treated and control PC-3 and C4-2B cells and showed that pso treatment resulted in increased nuclear localization of both p21 and p27 compared with vehicle-treated controls (Fig. 3E and F).

3.5. Overexpression of cdk4 or inhibition of p21 and p27 abrogates pso-induced G₀-G₁ arrest in CRPC cell lines

Because the most pronounced effect of pso was on cdk4 protein expression in both PC-3 and C4-2B cells, we asked whether ectopic overexpression of cdk4 rescinds pso-induced cell cycle arrest. Cells were transiently transfected with constitutively active cdk4 or empty vector, treated with pso, and cell cycle and western analyses were carried out. Western

analysis showed that pso treatment caused downregulation of endogenous cdk4 in empty vector-transfected PC-3 and C4-2B cells; however, no changes were observed in cdk4 expression in cells overexpressing cdk4 (Fig. 4A). Consistent with these results, pso treatment induced significant G₀/G₁ cell cycle arrest in vector-transfected cells, but failed to induce cell cycle arrest in cells overexpressing cdk4 (Fig. 4B).

Next, we investigated whether induction of p21 and p27 is required for pso-induced G₀/G₁ arrest. We transfected PC-3 cells with scrambled siRNA or p21 or p27 siRNA followed by treatment with pso (Fig. 5A and 5B). Flow cytometric analysis of cell cycle distribution showed that siRNA-mediated downregulation of p21 and p27 resulted in attenuation of pso-induced G₀/G₁ arrest compared to control siRNA-transfected PC-3 cells (Fig. 5B). Immunoblot analysis indicated that transfection of p21 and p27 siRNAs specifically knocked down p21 and p27 protein expression. Further, pso treatment did not induce p21 expression in PC-3 cells compared with scrambled siRNA-transfected cells (Fig. 5A).

4. Discussion

Synthetic, as well as naturally occurring, dietary compounds exhibit anticancer activity in CaP cells, and several cellular mechanisms underlying this effect have been identified. However, a number of studies supports the fact that cell cycle arrest, followed by induction of apoptosis, is the major molecular mechanism by which dietary phytochemicals exert their chemotherapeutic/chemopreventive effect [29,30]. Previous studies have shown that a furanocoumarin compound, pso, inhibits growth of CaP cells and induces apoptosis [7,25,38,39]. In the present study, we found that pso exerts growth-inhibitory effects in PC-3 and C4-2B CRPC cell lines by causing G₀/G₁ cell cycle arrest. Interestingly, no significant growth inhibition was seen in PBMCs, strongly suggesting that this effect of pso is specific to cancer cells and does not affect normal cells.

The progression of the cell cycle in eukaryotes is governed by complexes containing cyclins, the regulatory units, and cdks, the catalytic units. Cyclins D and E, together with cdk2, cdk4, or cdk6, play important roles in the progression of cells through the G₀/G₁ phase of the cell cycle [40]. Deregulation of G₀/G₁ phase cell cycle regulators is believed to promote the aberrant proliferation of cancer cells. Overexpression of cyclins and cdks can provide cancer cells with a selective growth advantage [14]. Therefore, targeting cyclin/cdk complexes is considered a promising and effective strategy for the treatment of CaP, and several cyclin-cdk inhibitory compounds are being tested in preclinical and clinical trials (reviewed in [12,15]).

The results obtained in the present study provide convincing evidence, for the first time, that pso exerts its effects on cell cycle progression primarily via inhibition of a cyclin/cdk complex. Pso treatment has a significant, time-dependent inhibitory effect on cyclin (D1, D2 and D3,) and cdk (2, 4, and 6) protein expression in both PC-3 and C4-2B cells. However, of these, expression of cdk4 was attenuated to the greatest extent by pso. Ectopic overexpression of cdk4, nevertheless, abrogated the effects of pso-induced G₀/G₁ arrest. Consistent with our results, a recent study showed that ectopic overexpression of cdk4 and cyclin D3 results in partial rescue from γ -secretase inhibitor-induced G₁ arrest in Notch-dependent T-cell lymphoma cell lines [22]. Hence, modulation of cdk4 expression could be another attractive target in the treatment of CRPC.

It is well established that the cyclin/cdk inhibitors p21 and p27 and the negative regulators INK4, p15, p16, and p18, play important roles in the regulation of cell cycle progression. Studies have shown that p21 and p27 are necessary for the assembly of the complexes of cyclin A with cdk4, cdk6, or cdk2 and of the cyclin E/cdk2 complex. Thus, p21 and p27

block cell cycle progression by inhibiting the activity of cyclin E/cdk2 complexes that normally promote G₁/S phase progression [9]. In the present study, we investigated the effect of pso on the expression of cdk inhibitors p21 and p27 in PC-3 and C4-2B cells and found that levels of p21 and p27 levels were markedly induced by pso in a time-dependent manner in both cell types. The increase in p21 and p27 levels was tightly correlated with G₀/G₁ phase arrest.

It is known that expression of p21 and p27 is regulated at transcriptional and post-transcriptional levels in different cell types [5,8]. We found that the pso-induced increases in p21 and p27 levels were mediated through upregulation of gene activity at the transcriptional level. The results of quantitative real-time RT-PCR showed a significant increase in p21, and a moderate increase in p27, mRNA expression. These results are in close correlation with the results of western analysis of p21 and p27 in both PC-3 and C4-2B cells treated with pso. Recent studies have shown that nuclear localization of p21 and p27 proteins is required for the inhibition of cdk activation by cdk-activating kinase [45]. In addition, localization of p21 and p27 in the nucleus is essential for controlling cell cycle progression [8,21]. In this study, we investigated the effect of pso on p21 and p27 subcellular localization and found that the p21 was significantly accumulated in the nucleus in pso-treated cells compared with its predominant localization in the cytoplasm in control cells. However, moderate nuclear accumulation of p27 was seen in pso-treated PC-3 and C4-2B cells, suggesting that the translocation of p21 to the nucleus may be involved in cell cycle arrest at G₀/G₁ phase.

To better comprehend the roles of p21 and p27, we examined the effects of pso in CRPC cell lines transfected with p21 and p27 siRNA. Ablation of p21 resulted in the attenuation of G₀/G₁ arrest, indicating that induction of p21 and p27 may be essential for psoinduced G₀/G₁ cell cycle arrest and growth inhibition in CRPC cell lines. These results are consistent with another study showing that siRNA-mediated ablation of p21 prevents growth arrest and apoptosis induced by the green tea polyphenol epigallocatechin-3-gallate in cancer cells [16].

The retinoblastoma tumor suppressor (Rb) family of proteins plays a key role in regulation of the cell cycle and downstream targets of G₀/G₁-specific cyclin/cdk complexes. In the G₀/G₁ phase, hypo-phosphorylated pRB (hypo-ppRB) binds to a transcription factor of the E2F family and suppresses its activity. E2F family proteins regulate the transcription of several genes whose products are required for either G₁/S transition or DNA replication. Thus, by negatively regulating E2F family proteins, pRB negatively controls cell cycle progression [17]. Upon exposure to a growth stimulus, the G₀/G₁-specific cyclin/cdks phosphorylate Rb proteins on multiple residues, causing the release of E2F transcription factors and promoting transcription of genes necessary for G₀/G₁ to S transition. Thus, pRB regulates the arrest and progression of the cell cycle based on its phosphorylation state [33]. We found that pso-mediated G₀/G₁ arrest in both PC-3 and C4-2B cells correlates, not only with the hypo-phosphorylation of Rb, but also with the inhibition of E2F1 protein expression.

Taken together, the results demonstrate that pso inhibits the growth of CRPC cell lines by inducing G₀/G₁ cell cycle arrest. The pso-mediated cell cycle arrest is associated with inhibition of cyclin/ cdk complexes and transcriptional regulation of p21 and p27 in CRPC cell lines. Further studies are warranted for the use of pso as a potential chemopreventive and chemotherapeutic agent for prostate cancer, particularly CRPC.

Acknowledgments

This study was supported by USPHS R01-AT002890 awarded by the NCCAM

References

1. Aggarwal R, Ryan CJ. Castration-resistant prostate cancer: targeted therapies and individualized treatment. *Oncologist*. 2011; 16:264–275. [PubMed: 21339259]
2. Amaral TM, Macedo D, Fernandes I, Costa L. Castration-resistant prostate cancer: mechanisms, targets, and treatment. *Prostate cancer*. 2012; 2012:327253. [PubMed: 22530130]
3. Benci K, Mandic L, Suhina T, Sedic M, Klobucar M, Kraljevic Pavelic S, Pavelic K, Wittine K, Mintas M. Novel coumarin derivatives containing 1,2,4-triazole, 4,5-dicyanoimidazole and purine moieties: synthesis and evaluation of their cytostatic activity. *Molecules*. 2012; 17:11010–11025. [PubMed: 22971585]
4. Berthet C, Klarmann KD, Hilton MB, Suh HC, Keller JR, Kiyokawa H, Kaldis P. Combined loss of Cdk2 and Cdk4 results in embryonic lethality and Rb hypophosphorylation. *Dev. Cell*. 2006; 10:563–573. [PubMed: 16678773]
5. Besson A, Gurian-West M, Chen X, Kelly-Spratt KS, Kemp CJ, Roberts JM. A pathway in quiescent cells that controls p27Kip1 stability, subcellular localization, and tumor suppression. *Genes Dev*. 2006; 20:47–64. [PubMed: 16391232]
6. Bloom J, Cross FR. Multiple levels of cyclin specificity in cell-cycle control. *Nat. Rev. Mol. Cell Biol*. 2007; 8:149–160. [PubMed: 17245415]
7. Bronikowska J, Szliszka E, Jaworska D, Czuba ZP, Krol W. The coumarin psoralidin enhances anticancer effect of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). *Molecules*. 2012; 17:6449–6464. [PubMed: 22643355]
8. Cmielova J, Rezacova M. P21Cip1/Waf1 protein and its function based on a subcellular localization [corrected]. *J. Cell Biochem*. 2011; 112:3502–3506. [PubMed: 21815189]
9. Coqueret O. New roles for p21 and p27 cell-cycle inhibitors: a function for each cell compartment? *Trends Cell Biol*. 2003; 13:65–70. [PubMed: 12559756]
10. Deep G, Agarwal R. New combination therapies with cell-cycle agents. *Curr. Opin. Invest. Drugs*. 2008; 9:591–604.
11. Denicourt C, Dowdy SF. Cip/Kip proteins: more than just CDKs inhibitors. *Genes Dev*. 2004; 18:851–855. [PubMed: 15107401]
12. Dickson MA, Schwartz GK. Development of cell-cycle inhibitors for cancer therapy. *Curr. Oncol*. 2009; 16:36–43. [PubMed: 19370178]
13. George D, Moul JW. Emerging treatment options for patients with castration-resistant prostate cancer. *Prostate*. 2012; 72:338–349. [PubMed: 21748753]
14. Hall M, Peters G. Genetic alterations of cyclins, cyclin-dependent kinases, and Cdk inhibitors in human cancer. *Adv. Cancer Res*. 1996; 68:67–108. [PubMed: 8712071]
15. Hallstrom TC, Nevins JR. Balancing the decision of cell proliferation and cell fate. *Cell Cycle*. 2009; 8:532–535. [PubMed: 19182518]
16. Hastak K, Agarwal MK, Mukhtar H, Agarwal ML. Ablation of either p21 or Bax prevents p53-dependent apoptosis induced by green tea polyphenol epigallocatechin-3-gallate. *FASEB J*. 2005; 19:789–791. [PubMed: 15764647]
17. Henley SA, Dick FA. The retinoblastoma family of proteins and their regulatory functions in the mammalian cell division cycle. *Cell Div*. 2012; 7:10. [PubMed: 22417103]
18. Higano CS, Crawford ED. New and emerging agents for the treatment of castration-resistant prostate cancer. *Urol Oncol*. 2011; 29:S1–S8. [PubMed: 22074657]
19. Ho E, Beaver LM, Williams DE, Dashwood RH. Dietary factors and epigenetic regulation for prostate cancer prevention. *Adv. Nutr*. 2011; 2:497–510. [PubMed: 22332092]
20. Hong WK, Sporn MB. Recent advances in chemoprevention of cancer. *Science*. 1997; 278:1073–1077. [PubMed: 9353183]
21. Jiang Y, Zhao RC, Verfaillie CM. Abnormal integrin-mediated regulation of chronic myelogenous leukemia CD34+ cell proliferation: BCR/ABL upregulates the cyclin-dependent kinase inhibitor, p27Kip, which is relocated to the cell cytoplasm and incapable of regulating cdk2 activity. *Proc. Natl. Acad. Sci. USA*. 2000; 97:10538–10543. [PubMed: 10973491]

22. Joshi I, Minter LM, Telfer J, Demarest RM, Capobianco AJ, Aster JC, Sicinski P, Fauq A, Golde TE, Osborne BA. Notch signaling mediates G1/S cell-cycle progression in T cells via cyclin D3 and its dependent kinases. *Blood*. 2009; 113:1689–1698. [PubMed: 19001083]
23. Kontogiorgis C, Detsi A, Hadjipavlou-Litina D. Coumarin-based drugs: a patent review (2008–present). *Expert Opin. Ther. Pat.* 2008; 22(2012):437–454. [PubMed: 22475457]
24. Kucuk O. Chemoprevention of prostate cancer. *Cancer Metastasis Rev.* 2002; 21:111–124. [PubMed: 12465750]
25. Kumar R, Srinivasan S, Pahari P, Rohr J, Damodaran C. Activating stress-activated protein kinase-mediated cell death and inhibiting epidermal growth factor receptor signaling: a promising therapeutic strategy for prostate cancer. *Mol. Cancer. Ther.* 2010; 9:2488–2496. [PubMed: 20736346]
26. Liang J, Zubovitz J, Petrocelli T, Kotchetkov R, Connor MK, Han K, Lee JH, Ciarallo S, Catzavelos C, Beniston R, Franssen E, Slingerland JM. PKB/Akt phosphorylates p27, impairs nuclear import of p27 and opposes p27-mediated G1 arrest. *Nat. Med.* 2002; 8:1153–1160. [PubMed: 12244302]
27. Lim DY, Tyner AL, Park JB, Lee JY, Choi YH, Park JH. Inhibition of colon cancer cell proliferation by the dietary compound conjugated linoleic acid is mediated by the CDK inhibitor p21CIP1/WAF1. *J. Cell Physiol.* 2005; 205:107–113. [PubMed: 15880444]
28. Mols F, Korfage IJ, Vingerhoets AJ, Kil PJ, Coebergh JW, Essink-Bot ML, van de Poll-Franse LV. Bowel, urinary, and sexual problems among long-term prostate cancer survivors: a population-based study. *Int. J. Radiat. Oncol. Biol. Phys.* 2009; 73:30–38. [PubMed: 18538503]
29. Pan MH, Ghai G, Ho CT. Food bioactives, apoptosis, and cancer. *Mol. Nutr. Food Res.* 2008; 52:43–52. [PubMed: 18080242]
30. Pan MH, Ho CT. Chemopreventive effects of natural dietary compounds on cancer development. *Chem. Soc. Rev.* 2008; 37:2558–2574. [PubMed: 18949126]
31. Riveiro ME, De Kimpe N, Moglioni A, Vazquez R, Monczor F, Shayo C, Davio C. Coumarins: old compounds with novel promising therapeutic perspectives. *Curr. Med. Chem.* 2010; 17:1325–1338. [PubMed: 20166938]
32. Sandal T. Molecular aspects of the mammalian cell cycle and cancer. *Oncologist.* 2002; 7:73–81. [PubMed: 11854549]
33. Shimizu-Sato S, Ike Y, Mori H. PsRBR1 encodes a pea retinoblastoma-related protein that is phosphorylated in axillary buds during dormancy-to-growth transition. *Plant Mol. Biol.* 2008; 66:125–135. [PubMed: 18034314]
34. Shore N, Mason M, de Reijke TM. New developments in castrate-resistant prostate cancer. *BJU Int.* 2012; 109(Suppl 6):22–32. [PubMed: 22672122]
35. Siegel R, Naishadham D, Jemal A. Cancer statistics. *CA Cancer J. Clin.* 2012; 62(2012):10–29. [PubMed: 22237781]
36. Simile MM, De Miglio MR, Muroli MR, Frau M, Asara G, Serra S, Muntoni MD, Seddaiu MA, Daino L, Feo F, Pascale RM. Down-regulation of c-myc and Cyclin D1 genes by antisense oligodeoxy nucleotides inhibits the expression of E2F1 and in vitro growth of HepG2 and Morris 5123 liver cancer cells. *Carcinogenesis.* 2004; 25:333–341. [PubMed: 14604889]
37. Singh S, Johnson J, Chellappan S. Small molecule regulators of Rb-E2F pathway as modulators of transcription. *Biochim. Biophys. Acta.* 2010; 1799:788–794. [PubMed: 20637913]
38. Srinivasan S, Kumar R, Koduru S, Chandramouli A, Damodaran C. Inhibiting TNF-mediated signaling: a novel therapeutic paradigm for androgen independent prostate cancer. *Apoptosis.* 2010; 15:153–161. [PubMed: 19851870]
39. Szliszka E, Czuba ZP, Sedek L, Paradysz A, Krol W. Enhanced TRAIL-mediated apoptosis in prostate cancer cells by the bioactive compounds neobavaisoflavone and psoralidin isolated from *Psoralea corylifolia*. *Pharmacol. Rep.* 2011; 63:139–148. [PubMed: 21441621]
40. Vermeulen K, Van Bockstaele DR, Berneman ZN. The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer. *Cell Prolif.* 2003; 36:131–149. [PubMed: 12814430]

41. Viallard JF, Lacombe F, Belloc F, Pellegrin JL, Reiffers J. Molecular mechanisms controlling the cell cycle: fundamental aspects and implications for oncology. *Cancer Radiother.* 2001; 5:109–129. [PubMed: 11355576]
42. Xiao G, Li G, Chen L, Zhang Z, Yin JJ, Wu T, Cheng Z, Wei X, Wang Z. Isolation of antioxidants from *Psoralea corylifolia* fruits using high-speed counter-current chromatography guided by thin layer chromatography–antioxidant autographic assay. *J. Chromatogr. A.* 2010; 1217:5470–5476. [PubMed: 20663508]
43. Yang HJ, Youn H, Seong KM, Yun YJ, Kim W, Kim YH, Lee JY, Kim CS, Jin YW, Youn B. Psoralidin, a dual inhibitor of COX-2 and 5-LOX, regulates ionizing radiation (IR)-induced pulmonary inflammation. *Biochem. Pharmacol.* 2011; 82:524–534. [PubMed: 21669192]
44. Yang YM, Hyun JW, Sung MS, Chung HS, Kim BK, Paik WH, Kang SS, Park JG. The cytotoxicity of psoralidin from *Psoralea corylifolia*. *Planta. Med.* 1996; 62:353–354. [PubMed: 8792669]
45. Yaroslavskiy B, Watkins S, Donnenberg AD, Patton TJ, Steinman RA. Subcellular and cell-cycle expression profiles of CDK-inhibitors in normal differentiating myeloid cells. *Blood.* 1999; 93:2907–2917. [PubMed: 10216085]
46. Das TP, Suman S, Damodaran C. Reactive oxygen species generation inhibits epithelial-mesenchymal transition and promotes growth arrest in prostate cancer cells. *Molecular carcinogenesis.* 2013 <http://dx.doi.org/10.1002/mc.22014>.

Fig. 1.

Pso inhibits cell growth and induces prominent G₀/G₁ cell cycle arrest in CRPC cell lines. (A) PC-3 and C4-2B cells and (B) PBMCs were treated with either DMSO or the indicated dose of pso for 24 h and the percentage of viable cells was determined using the Trypan blue exclusion method. (C) PC-3 cells were treated with vehicle or 25 μ M pso for 12, 24, and 48 h. (D) C4-2B cells were treated with vehicle or 30 μ M pso for 12, 24, and 48 h. The percentage of cells in each phase of the cell cycle was determined by flow cytometry. Values represent mean $\pm$ standard error of the mean of three independent experimental samples. ** $P < 0.05$ versus vehicle control.

Fig. 2. Effect of pso on expression of cell cycle regulatory proteins, Rb, and E2F in CRPC cell lines. PC-3 (left) and C4-2B (right) cells were treated with pso and lysates were prepared. Equal amounts (25 µg protein) of lysates were subjected to SDS-PAGE and western analysis, and expression patterns of (A) cyclins (D1, D2 and D3), (B) cdks (2,4, and 6), (C) cyclin/cdk inhibitors (p21 and p27), (D) pRb and total Rb, and (E) E2F1 were visualized.

Fig. 3. Pso induces transcriptional upregulation of p21 and p27 in CRPC cell lines. A and B PC-3 (left) and C4-2B (right) cells were treated with vehicle or pso for 24 h and C and D qPCR was carried out to determine expression levels of p21 and p27. Immunofluorescence analysis of p21 and p27 was carried out in (E) PC-3 and (F) C4-2B cells.

Fig. 4.

Ectopic overexpression of cdk4 rescued pso-induced G₀/G₁ arrest in CRPC cell lines. PC-3 (left) and C4-2B (right) cells were transfected with empty vector or plasmid encoding constitutively active cdk4 for 24 h and treated with pso for 24 h. (A) Lysates were prepared from G₀/G₁-arrested cells and proteins were separated by SDS-PAGE and probed with cdk4 antibodies. (B) Cell cycle distribution was analyzed by flow cytometry. Data are representative of two independent experiments.

Fig. 5. SiRNA knockdown of p21 and p27 in PC-3 cells, attenuates pso-induced G0/G1 phase arrest. (A). PC3 cells were transiently transfected with scrambled (SCR-siRNA), p21 siRNA, or p27 siRNA and treated with pso for 24h and cell lysates were subjected to Western blot analysis and (B) Cell cycle distribution was analyzed by flow cytometer. Data are representative of two independent experiments.