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ProstaCaid™ inhibits tumor growth in a xenograft model of human prostate cancer

JIAHUA JIANG[1], JAGADISH LOGANATHAN[1], ISAAC ELIAZ[2], COLIN TERRY[1], GEORGE E. SANDUSKY[3] and DANIEL SLIVA[1,4,5]

[1]Cancer Research Laboratory, Methodist Research Institute, Indiana University Health, Indianapolis, IN; [2]Amitabha Medical Clinic and Healing Center, Sebastopol, CA; Departments of [3]Pathology and [4]Medicine, [5]Indiana University Simon Cancer Center, Indiana University School of Medicine, Indianapolis, IN, USA

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Abstract. We have recently demonstrated that the dietary supplement ProstaCaid™ (PC) inhibits growth and invasive behavior of PC-3 human prostate cancer cells *in vitro*. In the present study, we evaluated toxicity and whether PC suppresses growth of prostate cancer in a xenograft model of human prostate cancer cells implanted in mice. Here, we show that an oral administration of PC (100, 200 and 400 mg/kg) did not affect body weight or activity of liver enzymes (ALT, AST) and did not show any sign of toxicity in liver, spleen, kidney, lung and heart tissues in mice. In addition, PC treatment resulted in the inhibition of tumor volumes (1024.6±378.6 vs. 749.3±234.3, $P<0.001$) in a xenograft model of prostate cancer with human hormone refractory (independent) PC-3 prostate cancer cells. Moreover, qRT-PCR analysis demonstrated significant upregulation of expression of *CDKN1A* (p21) and inhibition of expression of *IGF2*, *NR2F2* and *PLAU* (uPA) genes by an oral administration of PC in prostate cancer xenografts. Our study demonstrates that the concentrations of the dietary supplement ProstaCaid tested did not show signs of toxicity, and its oral application has significant anticancer activity *in vivo* and can be considered as an alternative treatment for prostate cancer patients.

Introduction

Prostate cancer is the most frequently diagnosed cancer among males in economically developed countries (1) and one of the leading causes of cancer-related deaths in men in the United States (2). In spite of the hormone ablation therapy and chemotherapy for advanced stage prostate cancer, these treatments do not show improving patient survival rate (3,4). Since experimental studies demonstrated that some natural/dietary compounds can prevent or treat prostate cancer in a variety of animal models, these natural agents can be employed for the alternative treatment of prostate cancer. Indeed, natural herbal and phytochemical agents are being recognized as an alternative therapy of prostate cancer patients (5).

ProstaCaid™ (PC) is a polybotanical dietary supplement which inhibits aberrant cell proliferation, induce apoptosis and inhibits invasiveness of a variety of prostate cancer cells, respectively (6,7). PC contains mycelium from Asian medicinal mushrooms (*Ganoderma lucidum*, *Coriolus versicolor* and *Phellinus linteus*), which separately demonstrated anticancer activities against prostate cancer cells *in vitro* and in animal experiments (8-13). In addition, some of the natural agents in PC demonstrated a variety of activities against prostate cancer cells. For example, curcumin from turmeric (*Curcuma longa*) induced apoptosis of prostate cancer cells and chemo- and radio-sensitized prostate tumors (14,15). Further, curcumin, quarcetin, resveratrol, baicalein (from *Scutellaria baicalensis*) and epigallocatechin-3-gallate (EGCG) inhibit prostate cancer cell growth and their combinations prevent tumorigenesis in a prostate cancer model in TRAMP mice (16). Moreover, β-sitosterol and vitamin C suppressed tumor growth and metastasis in hormone refractory prostate cancer (17,18) and 3,3'-diinodlylmethane (DIM) and lycopene inhibit prostate carcinogenesis in TRAMP mice, respectively (19,20). Finally, dietary supplementation with zinc or selenium protected against prostate carcinogenesis and increased survival in a mouse adenocarcinoma prostate model (21,22).

In the present study, we evaluated toxicity and anticancer activities of PC in an animal model of human hormone refractory (independent) prostate cancer cells PC-3 implanted in nude mice. Here, we show that PC is not toxic and its oral application suppresses growth of prostate tumors. In addition, PC modulates expression of *CDKN1A* (p21), *IGF2*, *NR2F2* and *PLAU* (uPA) genes in prostate cancer xenografts. Our results confirm that PC is not toxic and inhibits growth of human prostate cancer cells *in vivo*.

Materials and methods

Cell culture and reagents. The human prostate cancer cell line PC-3 was obtained from ATCC (Manassas, VA, USA). PC-3 cells were maintained in DMEM/F-12 medium

Correspondence to: Dr Daniel Sliva, Cancer Research Laboratory, Methodist Research Institute, 1800 N Capitol Ave, E504, Indianapolis, IN 46202, USA
E-mail: dsliva@iuhealth.org

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containing penicillin (50 U/ml), streptomycin (50 U/ml) and 10% fetal bovine serum (FBS). Medium and supplements came from Invitrogen (Grand Island, NY, USA). FBS was obtained from Hyclone (Logan, UT, USA). ProstaCaid (PC) a 33 ingredient comprehensive poly-herbal and nutrition preparation containing the following active weight components: *Curcuma longa* root extract complex with enhanced bioavailability (BCM-95®) 20%, quercetin 15%, *Coriolus versicolor*, *Ganoderma lucidum*, *Phellinus linteus* mushroom mycelium blend 10% [*Astragalus membranaceus* root extract (5:1), *Coix lacryma-jobi* seed extract (5:1), *Coptis japonica* rhizome extract (10:1), *Eleutherococcus senticosus* root extract (5:1), *Scutellaria baicalensis* root extract (5:1), *Scutellaria barbata* root extract (10:1), *Smilax glabrae* extract (5:1), *Taraxacum officinale* herb (5:1)] herbal blend 9%, *Urtica dioica* herb extract (5:1) 6%, β sitostererol 6%, *Serenoa repens* berry 5%, *Brassica oleracea* var. italic herb extract (22:1) 4%, *Punica granatum* fruit (40% ellagic acid) 4%, *Vitis vinifera* fruit skin extract (10:1) 4%, vitamin C 4%, α lipoic acid 3%, 3,3'-diinodlylmethane (DIM) 3%, *Cucurbita pepo* seed 2%, *Prunus africana* bark extract (4:1) 2%, *Camellia sinensis* herb extract (40% EGCG; 95% phenols; 70% catechins) 1.5%, lycopene 0.6%, Zinc 0.4%, vitamin D3 0.2%, resveratrol 0.2%, berberine 0.1%, boron 0.06%, selenium 0.004%, was supplied by the EcoNugenics, Inc. (Santa Rosa, CA, USA). PC stock solution was prepared by dissolving PC in sterile water and stored at 4˚C.

Toxicology studies. Toxicity of PC was evaluated in the 6 weeks old male nude mice (Harlan, Indianapolis, IN, USA). The mice were acclimatized for 1 week, and treated with PC (0, 100, 200 and 400 mg/kg of body weight, n=10 per group) by intragastrical gavage 5 times/week for additional 4 weeks. The body weight was evaluated three times per week. At the end of the experiment animals were euthanized by CO inhalation. Blood was collected and a gross pathology examination performed. Liver, spleen, kidney, lung and heart were harvested, fixed in 10% neutral-buffered formalin at 4˚C for 24 h followed tissue processing overnight and then embedded in paraffin. Five-micrometer sections were stained with hematoxylin and eosin (H&E). The levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), albumin and total protein were determined at the Department of Pathology and Laboratory Medicine, Indiana University (Indianapolis, IN, USA).

Human prostate tumor xenograft experiments. PC-3 cells ($3x10^6$) in 0.2 ml DMEM were implanted subcutaneously in the right flank on the ventral side of the 6 weeks old male nude mice (Harlan). After 1-2 weeks of implantation with tumor cells, when tumors reached ~20-30 mm^3, the animals were randomized into control and treatment groups (15 animals per group). The animals received intragastrical gavage 5 times/week with water (control) or 400 mg PC/kg of body weight (treatment) for additional 6 weeks The tumor size was measured using calipers and the tumor volume was estimated by the formula: tumor volume (mm^3) = $(LxW^2)x1/2$, where L is the length and W is the width of the tumor. At the end of the experiment (Day 42), the tumors were snap frozen and stored separately in liquid nitrogen. The protocol for animal experiments was approved by the Animal Research Committee at the Methodist Hospital (protocol no. 2010-05) according to the NIH guidelines for the Care and Use of Laboratory Animals.

Quantitative RT-PCR. The quantitative real-time polymerase chain reaction (qRT-PCR) was performed using the ABI PRISM 7900HT Fast Real-Time PCR System (Applied Biosystems) according to the manufacturer's instructions. Total RNA was isolated from tumors with RNAeasy (Qiagen, Valencia, CA). The RNA samples were reverse transcribed into cDNA (RT-PCR) using random hexamer primers and TaqMan reverse transcription kit (Applied Biosystems). The cDNA (100 ng per sample) was subjected to qPCR analysis in quadruplicate using forward and reverse primers, TaqMan Universal Master Mix and probe (10 ml per reaction) in fast optical 96-well plates. The data were analyzed using the ABI PRISM 7900 relative quantification (DDCt) study software (Applied Biosystems). In this study we have used primers *CAV1*, *CCND1*, *CDKN1A*, *E2F1*, *ELAC2*, *IGF2*, *MAPK6*, *NR2F2*, *PCNA* and *PLAU* genes with β-actin as internal control (Applied Biosystems). The gene expression levels are normalized to β-actin and are presented as arbitrary fold changes compared between control and treated groups.

Statistical analysis. Toxicology analyses of plasma were summarized using median (min, max) and compared across groups using Kruskal-Wallis tests and Mann-Whitney U tests with significance level adjusted using the Bonferroni correction. The changes in tumor volume in the control and treatment group were assessed by a random effects mixed model. Tumor weights were evaluated by the Mann-Whitney U test. RT-PCR data are presented as means ± SD and statistically evaluated by Student's t-test. The value of $P<0.05$ was considered to be significant.

Results and Discussion

PC is not toxic in vivo. Although recent studies suggested the beneficial effects of herbal supplements upon a variety diseases including cancer (23), all herbal/dietary supplements must be standardized and tested for their possible toxicity. In addition, recent study demonstrated that PC has the potency to kill prostate cancer cells by the induction of apoptosis (6). Therefore, to evaluate the toxicity of PC *in vivo*, male nude mice were orally gavaged by 0, 100, 200 and 400 mg/kg of body weight for 4 weeks as described in Materials and methods. Increased concentration of PC (100-400 mg/kg) did not significantly affect body weight of tested animals, suggesting that PC is not toxic (Fig. 1). In addition, gross necropsy did not show any sign of toxicity (data not shown) and H&E staining of control and highest PC dose treatment group (400 mg/kg) of liver, spleen, kidney, lung and heart did not demonstrate any abnormalities (Fig. 2). Moreover, liver enzyme profiles in plasma (ALT, AST and ALP) as well as albumin and total protein levels were not markedly changed (Table I), suggesting that PC is not hepatotoxic. Although some samples were not analyzed (e.g., control for ALP and total protein, 100 mg/kg of PC for albumin and total protein, Table I) because of the insufficient amount of collected blood, the levels for the treatment groups correspond to the previously determined levels of liver enzyme profiles in

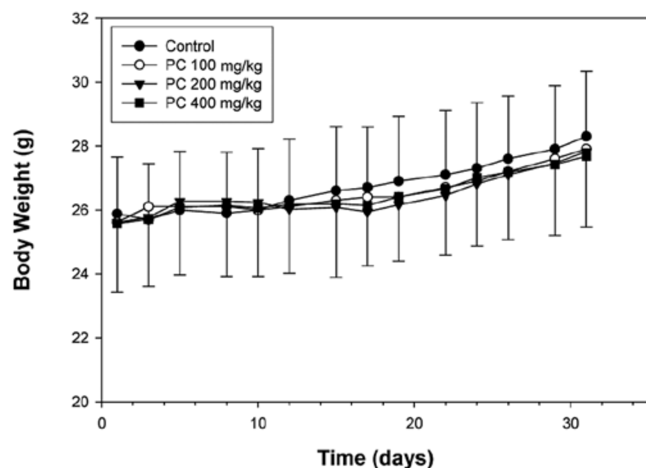

Figure 1. Effect of PC on body weight. Male nude mice were gavaged with PC (0, 100, 200 and 400 mg/kg of body weight, n=10 per group) 5 times/week for 4 weeks. The body weight was evaluated three times per week. Data are mean ± SD (SD showed for control and 400 mg PC/kg of body weight).

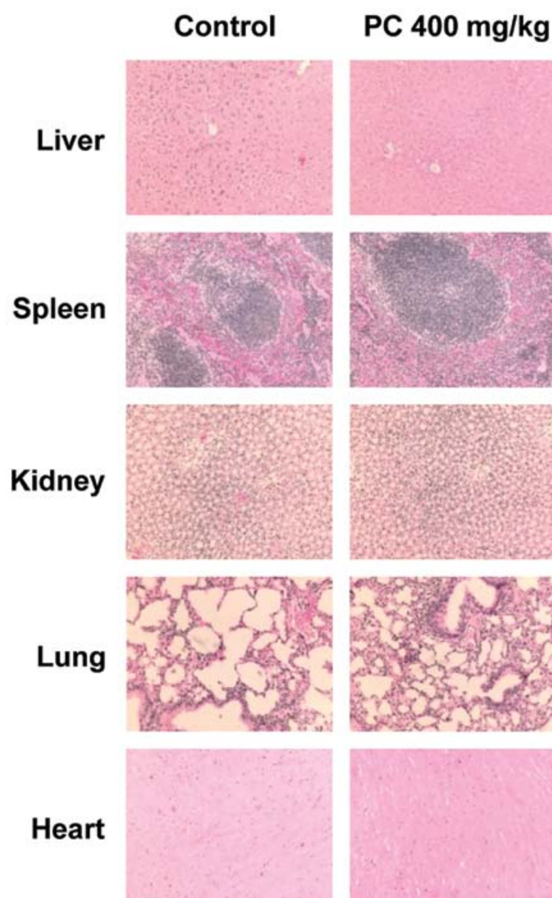

Figure 2. Histopathological evaluation. Mice were treated with PC as described in Fig. 1. Liver, spleen, kidney, lung and heart were harvested and stained with H&E as described in Materials and methods. The images are representative from control and high dose PC treatment (400 mg/kg) groups.

nude mice (24). In conclusion, dietary supplementation with PC (0-400 mg/kg) was not toxic in tested animals.

PC suppressed tumor growth in a xenograft model of human prostate cancer. Based on our data demonstrating that PC is not toxic *in vivo*, we have employed an animal model of human prostate PC-3 cancer cells xenografted in nude mice. PC-3 cells were subcutaneously injected into male nude mice, and when the forming tumors reached the size approximately 30 mm^3, the mice were divided into the control group (water) and the treatment group (400 mg PC/kg of body weight). There were no changes in the tumor volumes for the first 3 weeks of the treatment (Fig. 3A). However, after 3 weeks the tumors in the treatment group were smaller, and we observed a significant difference in the change in tumor volume over time between control and PC treatment groups (P<0.001). Although PC treatment decreased tumor weight from 0.75 g (0.36, 2.01; control) to 0.63 g (0.26, 1.32; PC treatment), this decrease was not significant (P=0.326, Fig. 3B). As mentioned above, different components of PC (e.g., extracts from *G. lucidum*, *C. versicolor*, *P. linteus* or isolated curcumin, quarcetin, resveratrol, baicalein and EGCG) suppressed prostate cancer in a variety of animal models. Therefore, the complex PC will allow reduced dosages of these isolated compounds and can therefore avoid the side effects associated with higher dosages

Table I. Effect of PC on liver enzymes.

	Control		PC (100 mg/kg)		PC (200 mg/kg)		PC (400 mg/kg)		
	n	Median (min, max)	n	Median (min, max)	n	Median (min, max)	n	Median (min, max)	P-value
Male mice									
ALT	5	65 (34, 117)	6	187.5 (65, 268)	9	168 (44, 446)	9	96 (50, 519)	0.120
AST	7	180 (110, 295)	6	267.5 (132, 525)	9	557 (86, 1149)	9	291 (119, 1124)	0.051
Alk Phos	0	N/A	2	119.5 (114, 125)	5	174 (117, 208)	8	132.5 (77, 169)	N/A
Albumin	2	1.3 (1.2, 1.3)	0	N/A	7	1.4 (1.4, 1.5)	9	1.4 (1.2, 1.4)	0.011
T protein	0	N/A	0	N/A	5	5.5 (5.2, 5.6)	4	5.5 (5.2, 5.6)	N/A

Data summarized using median (min, max) and compared across groups using Kruskal-Wallis tests. Comparisons of each group with its respective control performed using Mann-Whitney U tests with significance level adjusted using the Bonferroni correction. n, number of blood samples for which analyses were performed. N/A, not applicable.

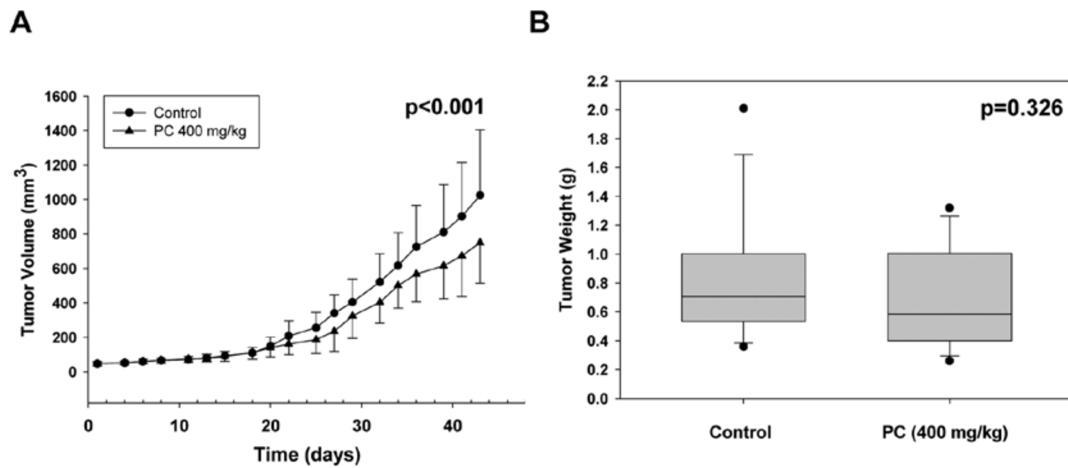

Figure 3. Effect of PC on the tumor size and tumor weight. PC-3 cells were injected subcutaneously into male nude mice and treated with water (control) or 400 mg PC/kg of body weight 5 times/week for daily for 42 days (n=15 mice per group) as described in Materials and methods. (A) Tumor volumes. Data are the mean ± SD and the changes in tumor volumes were statistically evaluated by a random effects mixed model ($P<0.005$). (B) Tumor weight. Data were analyzed by a non-parametric summary statistics (median, Q1, Q3) by the Mann-Whitney U test ($P=0.326$).

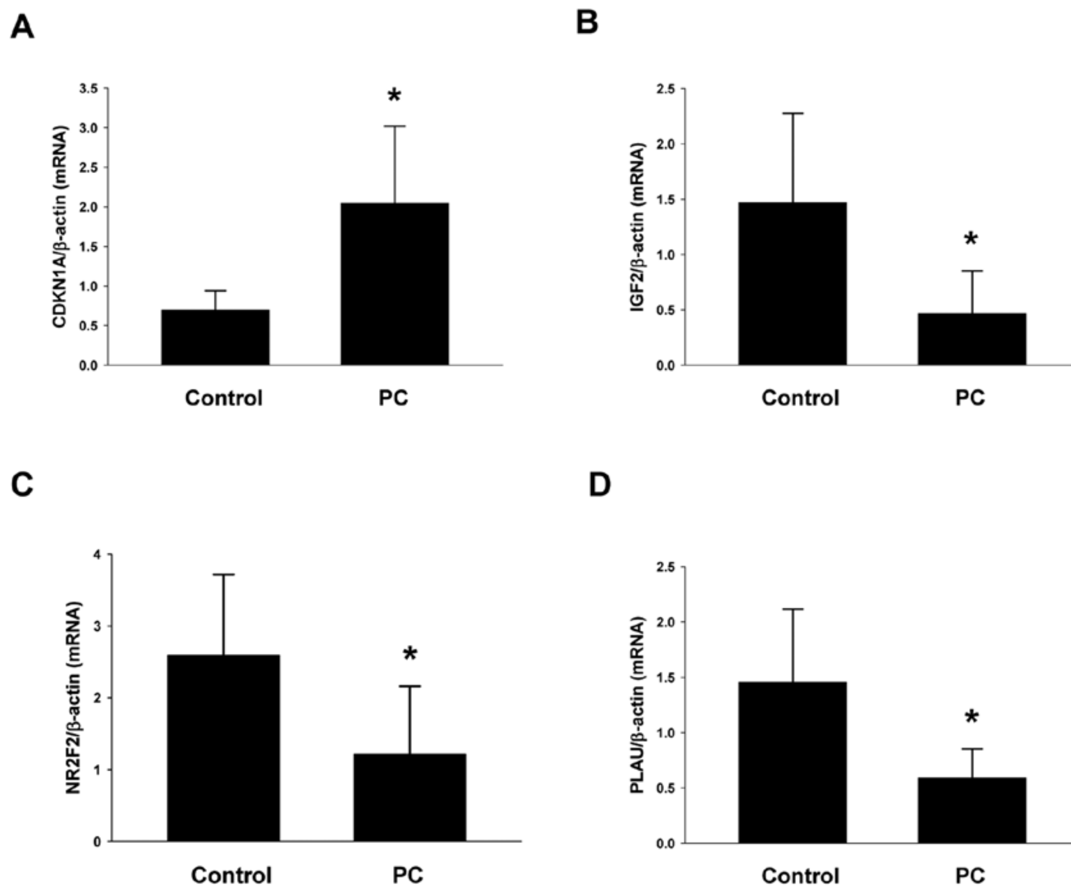

Figure 4. qRT-PCR in tumors. The mRNA expression of (A) CDKN1A, (B) IGF2, (C) NR2F2 and (D) PLAU in tumors from control and PC treatment (400 mg/kg) was determined by qRT-PCR as described in Materials and methods. The results are expressed as the relative expression ratios of specific mRNA to β-actin. Each bar represents the mean ± SD from 5 mice. *$P<0.05$ by t-test.

of the individual components. Indeed, another polybotanical agent TBS-101 (containing extracts from Panax ginseng, cranberry, green tea, grape skin, grape seed, *G. lucidum* and chamomile) was not toxic and suppressed tumor growth in mice (25). In addition, the use of different biologically active components in PC can also focus on the specific target molecules and signaling pathways which are associated with prostate cancer.

Effect of PC on the gene expression in tumors. Previous DNA-microarray analysis shown that PC upregulated the expression of *CDKN1A* and downregulated expression of *CAV1*, *CCND1*, *E2F1*, *ELAC2*, *IGF2*, *MAPK6*, *NR2F2* and *PLAU* genes in the PC-3 cells (7). To evaluate the effect of PC on the expression of these genes in prostate tumors, we isolated RNA and performed quantitative RT-PCR in control and PC treated mice as described in Materials and methods. In agreement with our *in vitro* study (7), PC treatment significantly increased expression of CDKN1A (p21) and downregulated expression of IGF2, NR2F2 and PLAU (uPA) (Fig. 4), whereas the expression of CAV1, CCND1, E2F1, ELAC2 and MAPK6 was not changed (data not shown).

The proliferation of cells is controlled by cyclins, cyclin-dependent kinases (CDKs) and CDK inhibitors (26). Therefore, one of the possible molecular targets in cancer cells is the activation of CDK inhibitors. Indeed, our observation that PC treatment significantly increased expression of CDK inhibitor CDKN1A-p21, is in agreement with recent studies demonstrating that some natural compounds (apigenin, silibinin, white cocoa tea extract) inhibited growth of PC-3 tumor xenografts and increased protein levels of p21 in these tumors (27-29).

An activation of autocrine IGF2 loop was originally linked to the neoplastic progression of prostate cancer (30). Furthermore, polymorphism of the *IGF2* gene and aging related epigenetic alteration such as loss of imprinting (LOI) for *IGF2* is associated with increased prostate cancer risk (31,32). Importantly, epigenetic inhibition of IGF2 expression suppressed hepatocellular carcinoma growth in nude mice (33), and a targeted gene therapy based on loss of IGF2 imprinting suppressed growth of colon tumor xenografts *in vivo* (34). Indeed, the downregulation of expression of IGF2 by PC in our study demonstrates the direct effect on the gene which overexpression is known to be involved in prostate cancer growth. Further studies are necessary to elucidate whether PC-induced downregulation of expression of IGF2 is mediated through epigenetic (e.g., promoter methylation) or other mechanisms.

NR2F2 (COUP-TFII) is a transcription factor originally described in the development of different organs such as heart, stomach, limb and skeletal muscles (34-38) and was recently identified as an inducer of mammary tumorigenesis and breast cancer metastasis *in vivo* (39). Moreover, NR2F2 expression was upregulated in the Epstein-Barr virus induced B-cell transformation (40). Interestingly, NR2F2 was overexpressed in the chemoresistant ovarian cancer tissues or cell lines (41), whereas NR2F2 expression was downregulated in chemosensitive oligodendroglial tumors (42). On the other hand, hypermethylation of NR2F2 promoter, associated with the downregulation of expression of NR2F2, was detected in tumor tissues from patients with human salivary gland adenoid cystic carcinoma (43) and mantle cell lymphoma (MCL) cells, respectively (44). Therefore, the function of NR2F2 in the development and progression of specific cancer depends on the cancer cell phenotype and its resistance or sensitivity to the particular treatment. In our study we show that PC treatment of tumors, developed from the androgen-resistant prostate cancer cells, downregulates expression of NR2F2 suggesting that NR2F2 is a suitable target for the hormone resistance prostate cancer.

Urokinase plasminogen activator (uPA; *PLAU* gene) is a crucial player in cancer metastasis and the invasive behavior (adhesion, migration and invasion) of cancer cells (23,45). Therefore, inhibition of expression of uPA abrogated prostate tumor growth in the orthotopic PC-3 tumors in nude mice (46). In addition, curcumin inhibited growth of prostate cancer LNCaP xenografts and suppressed expression of uPA (47). In agreement with our previous *in vitro* data with PC-3 cells (7), PC also suppressed expression of uPA in PC-3 xenografts. Therefore, our study confirms that inhibition of uPA *in vivo* results in the growth suppression of prostate cancer.

In conclusion, our data demonstrate that a novel dietary supplement ProstaCaid (PC) is not toxic *in vivo* at the amounts tested and inhibits growth of prostate tumors by the modulation of expression of p21, IGF2, NR2F2 and uPA. Our data suggest that PC has multiple targets and its special components may contribute to the inhibition of prostate cancer cells through distinct signaling pathways. In summary, PC may be considered as polybotanical preparation for the alternative therapy of prostate cancer.

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