Plumbagin inhibits prostate cancer development in TRAMP mice via targeting PKCe, Stat3 and neuroendocrine markers


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Plumbagin (PL), 5-hydroxy-2-methyl-1,4-naphthoquinone, is a quinoid constituent isolated from the roots of the medicinal plant Plumbago zeylanica L. (also known as chitrak). PL has also been found in Juglans regia (English Walnut), Juglans cinerea (black walnut) and Juglans nigra (black walnut). The roots of P. zeylanica have been used in Indian and Chinese systems of medicine for more than 2500 years for the treatment of various types of ailments. We were the first to report that PL inhibits the growth and invasion of hormone refractory prostate cancer (PCa) cells [Aziz, M.H. et al. (2008) Plumbagin, a medicinal plant-derived naphthoquinone, is a novel inhibitor of the growth and invasion of hormone-refractory prostate cancer. Cancer Res., 68, 9024–9032]. Now, we present that PL inhibits in vivo PCa development in the transgenic adenocarcinoma of mouse prostate (TRAMP). PL treatment (2mg/kg body weight i.p. in 0.2ml phosphate-buffered saline, 5 days a week) to FVB-TRAMP resulted in a significant (P < 0.01) decrease in prostate tumor size and genital apparatus weights at 13 and 20 weeks. Histopathological analysis revealed that PL treatment inhibited progression of prostatic intraepithelial neoplasia (PIN) to poorly differentiated carcinoma (PDC). No animal exhibited diffuse tumor formation in PL-treated group at 13 weeks, whereas 75% of the vehicle-treated mice elicited diffuse PIN and large PDC at this stage. At 20 weeks, 25% of the PL-treated animals demonstrated diffuse PIN and 75% developed small PDC, whereas 100% of the vehicle-treated mice showed large PDC. PL treatment inhibited expression of protein kinase C epsilon (PKCe), signal transducers and activators of transcription 3 phosphorylation, proliferating cell nuclear antigen and neuroendocrine markers (synaptophysin and chromogranin-A) in excised prostate tumor tissues. Taken together, these results further suggest PL could be a novel chemopreventive agent against PCa.

Introduction

Prostate cancer (PCa) continues to remain the most common cancer and the second leading cause of cancer-related deaths in American males. American Cancer Society has predicted that a total of 241,740 new cases of PCa will be diagnosed and 28,170 deaths will occur from it in the USA alone in the year of 2012 (1). Although PCa is frequently curable in its early stage by surgical or radiation therapy, many patients present locally advanced or metastatic disease for which there are currently no curative treatment option (2,3). Therefore, more effective therapies that can cure localized tumors and prevent progression of the disease are urgently needed. In recent years, chemoprevention by using nutraceuticals has become an ideal strategy to prevent or slowdown the various types of cancers (4,5) including PCa (6,7).

Abbreviations: NE, neuroendocrine; PCa, prostate cancer; PCNA, proliferating cell nuclear antigen; PIN, prostatic intraepithelial neoplasia; PKCe, protein kinase C epsilon; PL, plumbagin; Stat3, signal transducers and activators of transcription 3; Tag, T-antigen; TBS, Tris buffer saline; TRAMP, transgenic adenocarcinoma of mouse prostate.

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represents an ideal candidate disease for chemoprevention because of its age association and long latency period, and any modest delay achieved through pharmacological intervention could result in substantial reduction in the incidence of clinically detectable disease (6). We found that plumbagin (PL), a plant-derived quinoid (5-hydroxy-2-methyl-1,4-naphthoquinone), delays the invasive adenocarcinoma of prostate in the transgenic adenocarcinoma of mouse prostate (TRAMP) mouse model.

PL is an active constituent isolated from the roots of the medicinal plant Plumbago zeylanica L. [also known as Chitrak (8)]. PL has also been found in Juglans regia (English Walnut), Juglans cinerea (butternut and white walnut) and Juglans nigra [black walnut (8)]. The roots of P. zeylanica have been used in Indian and Chinese systems of medicine for more than 2500 years for the treatment of various types of ailments (8). PL has been shown for its potential health benefits including neuroprotective (9) and anticancer property against various types of cancers [Ref. (10) and references therein]. PL, fed in the diet (200 p.p.m.), inhibits azoxymethane-induced intestinal tumors in rats (11). PL inhibits ectopic growth of breast cancer MDA-MB-231 cells (12), non-small cell lung cancer A549 cells (13) and melanoma A375-S2 cells in athymic nude mice (14). Recently, we have shown that PL inhibits ultraviolet-radiation-induced development of squamous cell carcinomas (15). We including others have also reported its apoptosis inducing and growth inhibitory effects against pancreatic cancer (16,17) and PCa (18,19) cells. However, no study exists about the effects of PL in the prevention of prostate carcinogenesis in an intact mouse model. We present in this communication, for the first time, that PL administration inhibits progression of adenocarcinoma of prostate in the TRAMP mouse model, which is, in part, due to the inhibition of protein kinase C epsilon (PKCe), signal transducers and activators of transcription 3 (Stat3) activation and neuroendocrine (NE) markers (synaptophysin and chromogranin-A).

Materials and methods

Antibodies

Monoclonal or polyclonal antibodies specific for β-actin, chromogranin-A, synaptophysin, glyceraldehyde 3-phosphate dehydrogenase, PKCe, proliferating cell nuclear antigen (PCNA) and total Stat3 were purchased from Santa Cruz Biotechnology. Monoclonal antibodies specific for pStat3 Tyr705 and pStat3 Ser727 were obtained from BD Biosciences (San Jose, CA).

Experimental animals

Homozygous breeding pairs of TRAMP/FVB mice (congenic N20 strain), originally generated in Dr Allan Balmain’s laboratory, were provided by Dr Barbara Foster, Roswell Park Cancer Institute, Buffalo, NY. FVB/N mice were obtained from Harlan Sprague Dawley. Mice were screened for the presence of the SV40 large T-antigen (Tag) gene by PCR as detailed on the Jackson Laboratory website (http://jaxmice.jax.org/pub-cgi/protocols/protocols.sh?objtype=protocol&protocol_id=188). The animals were bred and maintained at the Animal Resources Facility of the University of Wisconsin-Madison. All of the animal protocols were approved by the University’s Research Animal Resources Committee in accordance with the NIH Guideline for the Care and Use of Laboratory Animals.

Study design

A total of 16 mice from TRAMP/FVB background were taken and divided into 2 groups with 8 animals in each group. At 4 weeks, PL administration (2mg/kg body weight, i.p. 5 days a week) in 0.2 ml of dimethyl sulfoxide and phosphate-buffered saline (1:10) ratio was given to one group of mice. Control group animals were treated with 0.2 ml of dimethyl sulfoxide and phosphate-buffered saline (1:10) ratio served as control. A total of eight mice, four animals from each group, were examined at 13 weeks of age and the remaining animals from each group were examined at 20 weeks for prostate tumor development. PL treatment was stopped 24h before killing the animals.

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Histopathological examination
Prostate tumors from both groups were excised and processed for histology as described previously (20). Dr Weixiong Zhong, MD, a certified pathologist, Department of Pathology, UW-Madison, examined all of the slides.

Western blot analysis
Portions of the excised PCa tissues from each group mice were used to prepare whole tissue lysates. In brief, PCa tissues were homogenized in lysis buffer [50 mmol/l N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid, 150 mmol/l NaCl, 10% glycerol, 1% Triton X-100, 1.5 mmol/l MgCl₂, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mmol/l phenylmethylsulfonyl fluoride, 200 µmol/l Na₃VO₄, 200 µmol/l NaF and 1 mmol/l EGTA (final pH 7.5)]. The homogenate was centrifuged at 14 000 g for 30 min at 4°C. Supernatants were collected and stored at −80°C until further use. Protein concentrations were estimated using Bio-Rad protein assay kit as per the manufacturer’s protocol. Fifty micrograms of cell lysate were fractionated on 10–15% Criterion precast sodium dodecyl sulfate-polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA). The fractionated proteins were transferred to 0.45 µm Hybond-P polyvinylidene difluoride transfer membrane (Amersham Life Sciences, Piscataway, NJ). The membrane was then incubated with the specific antibody followed by the appropriate horseradish peroxidase-conjugated secondary antibody (Thermo Scientific), and the detection signal was developed with Amersham’s enhanced chemiluminescence reagent using FOTO/Analyst Luminary Work Station (Fotodyne). The western blots were also imaged and quantitated by densitometric analysis using Total lab Nonlinear Dynamic Image analysis software (Nonlinear USA, Durham, NC).

Immunohistochemistry
To determine the expression of PKCε and PCNA proteins in vehicle and PL-treated PCa tissues, we performed immunohistochemistry in paraffin embedded sections (4 µm thickness). In brief, sections were deparaffinized by placing the slides at 60°C for 2h followed by three changes of xylene for 10 min each. Slides were placed in 63% methanol/hydrogen peroxide for 20 min for quenching endogenous peroxidase. Slides were rehydrated in one change of absolute, 95%, 75%, and 50% ethanol and distilled water. Antigen retrieval was performed by incubating samples at 116°C for 15 s in the declocking chamber by using a Tris-urea solution (pH 9.5). After antigen retrieval, tissue slides were incubated with 2.5% normal horse serum (R.T.U. Vectastain Universal Elite ABC Kit, Vector Laboratories) for 20 min to block non-specific binding of the antibodies. Subsequently, the slides were incubated over night with a mixture of PKCε (1:50) and PCNA (1:50) dilution in normal antibody diluents (Scy Tek # ABB-125) in a humidified chamber. Blocking peptides of PKCε were used to determine the specific immunoreactivity of these antibodies. We further confirmed the specificity of immunostaining of these proteins by using blocking peptide of PKCε and IgG antibody (served as a negative control). The mixture of antibodies was decanted and the slides were washed thrice in Tris buffer saline (TBS) (pH 7.4). The slides were incubated with appropriate secondary antibodies for 30 min at room temperature. Slides were rinsed with TBS for 5 min and ABC reagent (Vector kit) was applied for 30 min. Immunoreactive complexes were detected using diaminobenzidene substrate (Thermo Scientific) and counter stained with hematoxilin (Fisher Scientific) for nuclear staining. Finally, slides were mounted with a coverslip by using mounting medium, visualized under a Zeiss-Axiophot DM HT microscope and images captured with an attached camera.

Immunofluorescence
Paraffin-fixed excised prostate tumor tissue sections (4 µm thick) from control and PL-treated mice were used to determine the expression of chromogranin-A. After antigen retrieval by incubating samples at 95°C in Tris-urea solution

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**Fig. 1.** Effects of PL treatment on prostate tumor development in TRAMP mice. PL (2 mg/kg body weight, i.p., 5 days a week) or the vehicle treatment was started when the mice were 4 weeks old and stopped 24h before killing. There were eight mice per group. Mice were killed at ages 13 and 20 weeks. (A) Representative pictures of dissected vehicle and PL-treated TRAMP mice at age 13 weeks (AI) and 20 weeks (AII), respectively. (B) Representative pictures of excised genito-urinary tracts from vehicle and PL-treated mice at age 13 weeks (BI) and 20 weeks (BII), respectively. (C) Bar graphs illustrate weights of excised genito-urinary apparatus (CI) and prostate tumor (CII) of vehicle and PL-treated mice at age 13 and 20 weeks, respectively. Each value in the bar graph represents mean ± SD of four different mice. Student’s t-test was carried out to evaluate the significant difference (P < 0.01).
(pH 9.5) for 30 min, the tissue slides were incubated with normal horse serum (1:10 dilution) for 30 min to block non-specific binding of the antibodies. Subsequently, the slides were incubated overnight with chromogranin-A (1:50 dilution) primary antibody in a humidified chamber. The mixture of antibodies was decanted and the slides were washed thrice in 1× TBS (pH 7.4). The slides were incubated with rhodamine labeled secondary antibody for 1 h at room temperature in the dark. The solution of secondary antibodies was decanted and the slides were washed thrice with TBS for 5 min intervals each in the dark. Finally, the specimens were mounted with coverslips using a drop of mounting medium containing 4′,6-diamidino-2-phenylindole (Vector Lab, Burlingame, CA). All sections were examined with an Olympus Microscope attached with fluorescence detector.

Statistical analysis
Student’s t-test was carried out to determine the significance. The P value < 0.05 was considered as significant.

Results
PL treatment inhibits the development of prostate carcinogenesis in TRAMP mice
The autochthonous TRAMP model mimics in many ways human prostate carcinogenesis. Focal adenocarcinoma in TRAMP mice develops rapidly within 10–20 weeks. Carcinoma arises in the dorsal lateral lobe, which is considered analogous to the peripheral zone, where the human PCa originates (21). This model has been wildly used for the investigation of the molecular targets and their inhibitors for the development and metastasis of PCa (6,7,20,22). In our experiment, we used TRAMP/FVB mice. PL treatment (2 mg/kg body weight, i.p., 5 days a week) was started when mice were 4 weeks old. Vehicle group mice received 0.2 ml vehicle only. No apparent toxicity was observed with PL administration during the entire period of the experiment. All of the mice were examined at 13 and 20 weeks of age for prostate tumor development (Figure 1A and 1B). As shown in Figure 1, PL treatment, at both 13 and 20 weeks, elicited a significant (P < 0.01) decrease in tumor size (AII and BII) and weights of the genito-urinary tracts (CI) and prostate tumors (CII) compared to vehicle-treated animals.

PL treatment inhibits the progression of invasive adenocarcinoma in TRAMP mice
Histopathological analyses of the excised prostate tumor tissues of both control and PL-treated mice, at 13 and 20 weeks, are shown in Figure 2. Histopathological examination revealed that prostates from the control animals had mixed prostatic intraepithelial neoplasia (PIN) and poorly differentiated carcinoma (PDC) at 13 weeks (Figure 2AI). The PDC exhibited marked polymorphism and high levels of mitosis and apoptosis with NE phenotype of large nuclei. No apparent toxic symptoms were observed with PL administration during the entire period of the experiment. All of the mice were examined at 13 and 20 weeks of age for prostate tumor development (Figure 1A and 1B). As shown in Figure 1, PL treatment, at both 13 and 20 weeks, elicited a significant (P < 0.01) decrease in tumor size (AII and BII) and weights of the genito-urinary tracts (CI) and prostate tumors (CII) compared to vehicle-treated animals.

Fig. 2. Effects of PL treatment on the progression of invasive carcinoma in TRAMP mice. Histopathological analyses of excised prostate tumor tissues at age 13 and 20 weeks. (A) Representative photographs showing H&E staining of excised prostate tumors from vehicle (AI) and PL-treated (AII) animals at age 13 and 20 weeks, respectively. Black, green and red arrows indicate benign prostatic epithelium, PIN and poorly differentiated carcinoma, respectively. (B) Histopathological analysis results of excised prostate tumors from vehicle and PL-treated mice were summarized in the table.
with fine chromatin and inconspicuous nucleoli and scant cytoplasm with inapparent cell membranes (Figure 2AI–II). PL treatment inhibited progression of invasive adenocarcinoma in TRAMP mice. At 13 weeks, PL treatment resulted in focal PIN in 3 animals (Figure 2A and B), and diffuse PIN in one animal (Figure 2B). At the same time point (13 weeks), one of the four animals showed a small PDAC in the PL-treated group (Figure 2AII and B). At 20 weeks of age, prostates of PL-treated TRAMP mice contained focal or diffuse PIN (50% each). Although 50% of the PL-treated TRAMP mice illustrated PDAC, the size of the tumor was significantly \((P < 0.01)\) smaller than the vehicle-treated animals. These data suggest the chemopreventive potential of PL against progression of PCa.

PL treatment inhibits PKC\(\varepsilon\) and phosphorylated Stat3 expression in TRAMP mice

Expression levels of PKC\(\varepsilon\) and Stat3 correlate with PCa aggressiveness (22). We have previously reported that Stat3, which is constitutively activated in a wide variety of human cancers, including PCa, interacts with PKC\(\varepsilon\) (22). Results from the experiments, involving reciprocal immunoprecipitation and pull-down assays using recombinant PKC\(\varepsilon\) and Stat3, indicate that PKC\(\varepsilon\) mediates Stat3Ser727 phosphorylation is essential for both Stat3 DNA-binding and transcriptional activity (23). Also, PL preferentially inhibits PKC\(\varepsilon\) expression in PCa DU-145 cells \(\textit{in vitro}\) and their xenograft-derived tumors in athymic nude mice (18). To determine whether PL targets the expression of PKC\(\varepsilon\) by immunohistochemistry and western blot analysis in excised prostate tissues from control and PL-treated animals. PL treatment of TRAMP mice resulted in a decrease in PKC\(\varepsilon\) expression in excised prostate tumor tissues at 13 weeks (Figure 3AI) and 20 weeks (Figure 3AII) compared with vehicle-treated animals. These results were further confirmed by western blot analysis. As shown in Figure 3BI–II, PL treatment inhibited the expression of PKC\(\varepsilon\) at both 13 and 20 weeks, respectively, compared with vehicle-treated animals. At 20 weeks, PL showed a more pronounced effect compared with 13 weeks. In the same experiment, PL treatment inhibited phosphorylation of
both Stat3Tyr705 and Stat3Ser727 at 13 and 20 weeks in prostate tumor tissues compared with vehicle-treated mice (Figure 3BI–II). No significant effect was observed on the protein levels of total Stat3 (Figure 3AI–II).

**PL treatment inhibits expression of PCNA in TRAMP mice**

PCNA is one of the cell proliferative markers that overexpressed in the S phase of cell cycle (24). PCNA has also been considered as prognostic biomarkers for various types of cancers including PCa (25,26). We observed that deletion of PKCε in TRAMP mice inhibits the expression PCNA (20). Here, we analyzed the effect of PL on the expression of PCNA. Immunohistochemical analysis indicated that PL treatment inhibited expression of PCNA in PIN of excised prostates compared with vehicle-treated mice (Figure 3C).

**PL treatment inhibits the expressions of synaptophysin and chromogranin-A in TRAMP/FVB mice**

The study of the influence of genetic background in prostate pathology between FVB and B6 mice revealed that FVB mice have a significantly higher incidence of malignant NE carcinomas and shorter survival time compared with B6 mice (27). All FVB mice develop malignant NE carcinomas by 20 weeks compared with lifetime incidence of 20% in B6 mice (27). PL treatment effectively inhibited the development of NE carcinomas in TRAMP/FVB mice (Figure 4BIII). These findings prompted us to determine the effect of PL on the expression of NE markers (synaptophysin and chromogranin-A) in excised prostate tumor tissues. Chromogranin-A expression level in control mice with predominantly large was much higher at 20 weeks compared with 13 weeks (Figure 4AI–II). However, synaptophysin expression was detected at both 13 and 20 weeks (Figure 4AI–II). PL-treated mice had predominantly PIN and small PDA and exhibited low levels of both synaptophysin (Figure 4AI–II) and chromogranin-A (Figure 4AI–II) compared with vehicle-treated animals. We also confirmed these findings by immunofluorescence. Results further demonstrated inhibition of chromogranin-A expression at 20 weeks (Figure 4BI–II).

**PL treatment does not affect the expression of transgene SV40 Tag**

A spontaneous development of PCa in male TRAMP mice is mediated by expression of SV40 Tag oncoprotein targeted by the rat probasin promoter, which is highly specific to the prostate epithelial cells (21). A possibility was explored whether the inhibition of PCa development in TRAMP/FVB mice by PL is the result of inhibition of Tag. In this experiment (Figure 4CI–II), the effect of PL treatment on the expression of Tag in PCa was analyzed at both 13- and 20-week-old mice. PL treatment did not inhibit Tag expression in PCa.

**Discussion**

There is an urgent need for agents, which are effective and selective, in the prevention and/or treatment of PCa development and metastasis. We have previously reported that: (i) PL is a unique plant-derived chemopreventive agent, which selectively inhibits expression of PKCε, a predictive biomarker of PCa progression; (ii) PL administration delays ectopic growth of hormone refractory PCa cells as well as reduces both tumor weight and volume by 90%; (iii) PL inhibits invasion of PCa cells. We now present that PL inhibits PCa development in TRAMP mice via targeting PKCε, Stat3 and NE markers.

PL has been shown to exert antitumor and antiproliferative activities in animal models and in cell culture (9). A few examples will be cited. Recently, we have shown that topical application of PL (100–500 nmol) prevents ultraviolet-radiation-induced cutaneous squamous cell carcinoma (19). We have also reported that PL administration (2 mg/kg body weight dose i.p.) prevents the growth of PCa.
(DU-145) and pancreatic cancer Panc1 cells derived tumors in xenograft models (21,22). In the present study, we observed chemopreventive effects of PL against PCa. Our results indicate that PL treatment inhibits PCA carcinogenesis in TRAMP mice, which was evident from a significant (P < 0.01) decrease in the tumor weight (Figure 1C–II) and delay in the transformation of PIN to PDC in TRAMP mice (Figure 2A and 2B).

The mechanism by which PL inhibits the development of PCa involves multiple targets including PKCe (18). Evidence suggests that PKCe is an oncogene and plays an important role in the induction and progression of various types of cancers (28–30) including PCA (20,22,31). Overexpression of PKCe is sufficient to promote conversion of PCA androgen-dependent LNCaP cells to androgen-independent variant, which rapidly initiates tumor growth in vivo in both intact and castrated athymic nude mice (32). We have shown previously that PKCe expression level correlates with the aggressiveness of human PCA (22). Also, PKCe is overexpressed in PCA in TRAMP mice (22). Recently, we have shown that genetic loss of PKCe in TRAMP mice prevents development and metastasis of PCa (20). A recent study suggests that overexpression of PKCe in mouse prostate epithelium develops PIN at 16–18 weeks (33). Our data suggested a decrease expression of PKCe in the excised prostate tumor tissues of PL-treated animals (Figure 3A–II).

PKCe has also been linked to the regulation of various signaling pathways in PCa. A recent study has shown that overexpression of PKCe protected human LNCaP cells against apoptotic stimuli via inducing phosphorylation of Bad at Ser112 residue (34). It has been shown that integrin signaling links PKCe to the PKB/Akt survival pathway in recurrent human PCA cells (35). Proteomic analysis of human PCA CWR22 cell xenografts shows that the association of PKCe with Bax may neutralize apoptotic signals propagated through the mitochondrial death-signaling pathway (36). We have also shown that PKCe associates with Stat3 and this association increases with the PCa development and progression in human and TRAMP mouse model (22). We have also shown PKCe association with Stat3 in other human cancer cell lines and targeted deletion of PKCe by using specific siRNA inhibits Stat3 phosphorylation at Ser727 in these cancer cell lines (23). In TRAMP mice, we have shown that genetic loss of PKCe inhibits both Ser727 and Tyr705 Stat3 phosphorylation (20). These results prompted us to explore the effect of PL treatment on Stat3 activation in prostate tumor tissues of TRAMP mice. Our results demonstrated that PL treatment inhibits Stat3 activation in prostate tumors of TRAMP mice. We observed inhibition of Stat3 phosphorylation at both Ser727 and Tyr705 (Figure 3B–II). These findings are in accordance to our previous published report (18) and suggest that PL also targets PKCe and Stat3 in intact mouse model. Accumulating evidences now suggested that PKCe is a hallmark in the development of prostate carcinogenesis, which regulates multiple signaling pathways during the disease progression. We and others have reported that PKCe also regulates P13K/AKT/NF-κB/IL-6/EGFR signaling pathways (20,33,34). It might be possible that PL directly or indirectly through PKCe inhibits these signaling pathways in PCa. Further study is warranted in this direction. PL treatment also inhibited the cell proliferation marker PCNA. These findings are in accord with our previous published reports (15,17,18) and further suggest the strong antiproliferative effects of PL against PCA. NE prostate cancer is one of the aggressive subtypes of PCa in human, which generally arises after androgen ablation therapy (37). NE prostate cancer frequently metastasizes to visceral organs, responds only transiently to chemotherapy and most patients survive ≤1 year (37). Interestingly, we observed that PL treatment also inhibited the expression of well-known NE markers (synaptophysin and chromogranin-A) in TRAMP mice (Figure 4A and 4B). These data suggest that PL could also be helpful for the treatment of NE PCa.

In summary, overwhelming evidence suggests that PKCe is an oncogene, which is involved in the development, emergence of androgen independent, as well as metastasis of human PCa. Our results clearly indicate that PL prevents prostate tumor growth and progression in TRAMP mice model. Antitumor potential of PL in TRAMP mice could be partly due to the inhibition of PKCe, Stat3 and NE markers (synaptophysin and chromogranin-A). These results provide evidence that PL is a plant-derived chemopreventive natural agent, which delays the progression of PCa. We conclude that PL could be used as a potential chemopreventive agent for the treatment of human PCa.

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**References**


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