Plumbagin Inhibits Prostate Carcinogenesis in Intact and Castrated PTEN Knockout Mice via Targeting PKCɛ, Stat3, and Epithelial-to-Mesenchymal Transition Markers

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Abstract

Prostate cancer continues to remain the most common cancer and the second leading cause of cancer-related deaths in American males. The Pten deletions and/or mutations are frequently observed in both primary prostate cancers and metastatic prostate tissue samples. Pten deletion in prostate epithelium in mice results in prostatic intraepithelial neoplasia (PIN), followed by progression to invasive adenocarcinoma. The Pten conditional knockout mice ([Pten-laplox:PB-Cre⁺] [Pten-KO]) provide a unique preclinical model to evaluate agents for efficacy for both the prevention and treatment of prostate cancer. We present here for the first time that dietary plumbagin, a medicinal plant–derived naphthoquinone (200 or 500 ppm) inhibits tumor development in intact as well as castrated Pten-KO mice. Plumbagin has shown no signs of toxicity at either of these doses. Plumbagin treatment resulted in a decrease expression of PKCɛ, AKT, Stat3, and COX2 compared with the control mice. Plumbagin treatment also inhibited the expression of vimentin and slug, the markers of epithelial-to-mesenchymal transition (EMT) in prostate tumors. In summary, the results indicate that dietary plumbagin inhibits growth of both primary and castration-resistant prostate cancer (CRPC) in Pten-KO mice, possibly via inhibition of PKCɛ, Stat3, AKT, and EMT markers (vimentin and slug), which are linked to the induction and progression of prostate cancer. Cancer Prev Res; 8(5): 375–86. ©2015 AACR.

Introduction

Prostate cancer continues to remain the most common cancer and the second leading cause of cancer-related deaths in American males. The American Cancer Society predicted that a total of 233,000 new cases of prostate cancer would be diagnosed and 29,480 deaths would occur from it in the United States alone in the year 2014 (1). Prostate cancer may be curable in its early stage by surgical or radiotherapy but there are currently no curative treatment options available for advanced or castration-resistant prostate cancer (CRPC; refs. 2, 3). The FDA has approved two chemopreventive drugs, finasteride and dutasteride, which have been shown to reduce the risk of prostate cancer up to 25% in large clinical trials but both drugs showed potential side effects (1). Therefore, more effective therapies that can prevent or treat advanced or CRPC are urgently needed. In recent years, chemoprevention by using nutraceuticals has become an ideal strategy to prevent or slow down the various types of cancers (4, 5), including prostate cancer (6, 7).

Plumbagin, a plant-derived quinoid (5-hydroxy-2-methyl-1,4-naphthoquinone), isolated from the roots of the medicinal plant Plumbago zeylanica L. (also known as Chitrak; ref. 8) has been shown as a chemopreventive and therapeutic agent against various types of cancer, including prostate cancer. Plumbagin has also been found in Juglans regia (English Walnut), Juglans cinerea (butternut and white walnut), and Juglans nigra (blacknut; ref. 8). The roots of Plumbago zeylanica have been used in Indian and Chinese systems of medicine for more than 2,500 years for the treatment of various types of ailments (8). It has also been reported for its neuroprotective (9) and cardioprotective activities in mice (10). Plumbagin fed in the diet (200 ppm) inhibits azoxymethane-induced intestinal tumors in rats (11). Plumbagin inhibits ectopic growth of breast cancer MDA-MB-231 cells (12), azoxymethane-induced intestinal tumors in rats (11). Plumbagin 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). It has been reported that plumbagin inhibits osteoclastogenesis induced by breast cancer cells in mice (15). A recent study has also shown that plumbagin inhibits osteoclast formation and breast cancer cell–derived tumors in the bone microenvironment of mice (16). We previously have shown that plumbagin inhibits ultraviolet radiation–induced development of squamous cell carcinomas (17). We have also shown that plumbagin administration inhibits pancreatic cancer cell growth in vitro and in vivo via targeting EGFR, NF-kB, and Stat3 signaling pathways (18). Another study has also shown inhibition of pancreatic cancer cell–derived orthotopic xenograft tumors by plumbagin (19). Our laboratory has previously reported that plumbagin administration inhibits human prostate...
cancer cells DU-145 ectopic xenograft tumors (20). Recently, we have reported that plumbagin administration inhibits growth and metastasis of highly aggressive human prostate cancer cells (PC-3M; ref. 21) and prostate carcinogenesis in the transgenic adenocarcinoma of the mouse prostate (TRAMP; ref. 22). We now present in this communication for the first time that dietary administration of plumbagin inhibits prostate tumor growth in an intact as well as in a castrated Pten-KO mouse model possibly via inhibition of PKCε, Stat3, Akt, and epithelial-mesenchymal transition (EMT) markers (Vimentin and Slug).

Materials and Methods

Chemicals and antibodies

Plumbagin (practical grade, purity >95%) was purchased from Sigma-Aldrich. Monoclonal or polyclonal antibodies specific for AKT, β-actin, PKCε, and total Stat3 were purchased from Santa Cruz Biotechnology. Blocking peptide for PKCε, antibodies, and mouse IgG were also procured from Santa Cruz Biotechnology. Monoclonal antibodies specific for pAKT, pStat3 Tyr705, and pStat3 Ser727 were obtained from BD Biosciences. Vimentin and Slug antibodies were purchased from Cell Signaling Technology Inc.

LC-MS/MS assay

Fifty microliters of either the mouse plasma sample or plasma standard was placed in a microfuge tube. Ten microliters of working internal standard (50 ng/mL honokiol) was added in the tube and vortexed for one minute. One milliliter ethyl acetate was added in the tube and further vortexed for 10 minutes. The tube was centrifuged for 10 minutes at 14,000 rpm. The upper organic phase was transferred to a tube and evaporated under N2. The residue was reconstituted with 150 μL of 60% acetonitrile and placed on an autosampler plate. A 7-point plasma standard curve spanning the range 15.62 to 1,000 ng/mL was included with each set of samples. The high-performance liquid chromatography (HPLC) consisted of a model 1200 binary pump, vacuum degasser, thermostatted column compartment held at 25.0°C, and a model 1100 thermostatted autosampler held at 25.0°C, all from Agilent Technologies. The HPLC was coupled directly to a model API 4000 triple quadrupole mass spectrometer equipped with a Turbo V atmospheric pressure ionization source (API 4000 triple quadrupole mass spectrometer equipped with a Turbo V atmospheric pressure ionization source fitted with the electrospray probe from Applied Biosystems/MDS Sciex. A 150×4.6 mm Zorbax Extend C18 S5 micron HPLC column (Agilent) was the analytical column. The injection volume was 20 μL. The mobile phase solvents were: (A) Millipore Type I water and (B) HPLG grade acetonitrile. The solvents were mixed 40%/60%/60% B and delivered isocratically at 800 μL/minute. Run time was 10 minutes. Mass spectrometry data were obtained in negative ion mode. The multiple reaction monitoring (MRM) transitions were m/z 187 → m/z 159 for plumbagin and m/z 265.3 → m/z 244.1 for the internal standard honokiol. The retention time for plumbagin was approximately 4.8 to 5.9 minutes for honokiol. The lower limit of quantitation (LLOQ) for plumbagin was 15.62 ng/milliliter.

Generation of the Pten-loxp/loxP:PB-Cre4 (Pten-KO) mouse

Mice were generated in our laboratory by crossing Pten floxed (loxP/loxP) with Probasin-Cre (PB-Cre4−) as described (23). Both of the mice were on the C57/BL6J background. Pten floxed (loxP/loxP) mice from Jackson Laboratories were screened for the floxed 328 bp band and/or wild-type 156 bp band by using the Fwd IMR9554: caa gca ctc tgc gaa cag and Rev IMR9555: aag ttt tgt aag gca aga tgc. Probasin-Cre (PB-Cre4−) from the NCI Mouse Repository was screened for the 393 bp transgene by using the following primers: Fwd P021: ctt aag aat ggg aca ggc agg tatt g and Rev C031: cat cac tct tgt cat cga cc. The animals were bred and maintained at the Animal Resources Facility of the University of Wisconsin-Madison (Madison, WI). All of the animal protocols were approved by the University's Research Animal Resources Committee in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals.

Study design to determine the effects of plumbagin on the development of prostate cancer in intact Pten-KO mice

A total of 100 intact Pten-KO mice were used to determine the effect of plumbagin on prostate tumor growth. Mice were divided into three groups control (n = 40), plumbagin (200 ppm; n = 20), and plumbagin (500 ppm; n = 40). Plumbagin treatment was started at 4 weeks of age and continued until the mice were sacrificed. Plumbagin was mixed with the powder diet (8604 Harlen Tekland Rodent Diet) in a food processor for 10 minutes and poured into a glass cup and replaced with a fresh plumbagin-mixed diet at every alternate day. The control group of mice was fed with diet alone. Mice were sacrificed at 15 and 30 weeks and examined for prostate tumor growth.

Study design to determine the effects of plumbagin on the growth of prostate cancer in castrated Pten-KO mice

A total of 26 mice were used to determine the effect of plumbagin on CRPC in Pten-KO mice. At 10 weeks of age, all of the mice were castrated by removal of both of the testicles following the standard operation procedure. All of the protocols were approved by our University Institutional Animal Care Use Committee (IACUC). Three days after castration, the mice were divided into two groups (10 mice in each group) and were fed with the normal antioxidant-free diet and the diet containing plumbagin as described previously. In a parallel group, 10 intact Pten-KO mice were given the normal diet and served as control for castrated Pten-KO mice. All of the mice from both groups were sacrificed at 50 weeks, and the prostates were dissected and weighed. Part of the prostate tumor tissues were processed for tissue sectioning.

CT/PET imaging

Computed tomography/positron emission tomography (CT/PET) imaging of three untreated and three plumbagin treated Pten-KO mice were performed using a tumor specific radiopharmaceutical agent (131I-NM404). Details of the method for CT/PET imaging were described previously (24).

Histopathological examination

Part of the prostate from the control and plumbagin-treated mice was excised and processed for histology as described previously (25). Dr Weixiong Zhong (Pathologist, Department of Pathology, University of Wisconsin-Madison (Madison, WI), examined all the slides for histopathology.

Western blot analysis

Portions of the excised prostate cancer tissues from each group of mice were used to prepare whole tissue lysates as described (21). Fifty micrograms of cell lysate were fractionated on 10% to
15% Criterion precast SDS-polyacrylamide gels (Bio-Rad Laboratories).

**Immunofluorescence**

Paraffin-fixed prostate tumor tissue sections (4 µm thick) from control and plumbagin-treated mice were used to determine the expression of vimentin and slug. Detail methods of immunofluorescence are described previously (21). All of the sections were examined with an Olympus Microscope attached with fluorescence detector.

**PKCε kinase activity**

It was analyzed by the PKC kinase activity kit obtained from ENZO Life Science. The procedure followed the manufacturer’s protocol with slight modification. Forty microliters of specific phospho PKCε antibody were used in each assay sample containing 2 µg protein of excised prostate tumor tissue. Three individual prostate tumor tissue samples of each group at 15 and 30 weeks were analyzed. Protein from normal wild-type mouse prostate tissue and PKCε transgenic mouse epidermis were used as negative and positive controls, respectively. Data in the bar graph represent the mean ± SE. A P value of <0.05 was considered as significant.

**Cytokines array analysis**

Mouse-specific cytokines array analysis was performed in the serum of wild-type (WT), Pten-KO control, and plumbagin-treated Pten-KO groups. It was done by a commercially available facility (Eve Technologies Corporation). In brief, blood was collected from the retro-orbital plexus of WT, Pten-KO control, and plumbagin-treated Pten-KO groups. Serum was isolated by centrifugation of the blood at 5,000 rpm at 4°C and stored at −80°C until it was sent for analysis. The serum of 3 mice from each group was sent for analysis. Cytokines concentrations in mice serum were expressed in pg/mL.

**Statistical analysis**

Prostate and urogenital organs were excised and weighed upon sacrifice. The normality of the prostate weight data was assessed; log-transformed values were found to conform to normality assumptions better than raw values and subsequent analyses used log-transformed measures. Relationships between treatment arms and continuous data such as prostate weights were tested using the Wilcoxon rank-sum test where there were two treatment arms (0 ppm and 500 ppm), and with the Jonkheere–Terpstra test where there were three treatment arms (0 ppm, 200 ppm, and 500 ppm). Association between treatment and dichotomous data such as PIN or carcinoma differentiation was tested with...
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the Cochran-Armitage test for trend. Computations were performed with SAS (1A) and R software (2A) and figures were created with R software (21).

Results

Oral dose finding and bioavailability study of plumbagin in C57BL/6 wild-type mice

In this experiment (Fig. 1A), 20 (6 weeks old) wild-type (C57BL/6) mice were used. Mice were randomized into five different cohorts (i.e., 4 treatment cohorts and 1 control cohort). Mice were fed with plumbagin (500, 1,000, 2,000, 4,000 ppm) in the diet for 6 weeks and evaluated for weekly body weight change. Body weights of the plumbagin-fed mice were compared with the control mice. Body weight loss greater than 15% was considered as visual sign of toxicity in mice. Results indicate that plumbagin fed in diet was tolerable up to 2,000 ppm in wild-type mice (Fig. 1A). However, mice fed 4,000 ppm of plumbagin exhibited visual toxicity, which was observed by loss of body weight (Fig. 1A). We next evaluated the bioavailability of plumbagin in mouse plasma by the liquid chromatography/tandem mass spectrometry (LC-MS/MS) method as described (26). Plumbagin was detected approximately 50 ng/mL in the plasma of mice fed 2000 ppm dose. However, plumbagin was not detected in the plasma of mice given 500 ppm dose (Fig. 1Ai and ii).

Plumbagin treatment inhibits prostate tumor growth in intact Pten-KO mice

Prostate-specific conditional Pten-KO mice provide a unique model to define the mechanism of resistance to androgen ablution therapy in a genetically defined model where the initiating oncogenic event is not androgen dependent. These mice have a significantly shortened latency of prostatic intraepithelial neoplasia (PIN) formation, which results in prostate cancer progression to a metastatic stage, mimicking the disease progression in humans (23). We investigated whether dietary administration of plumbagin inhibits prostate tumor development in Pten-KO mice. In this experiment, 100 intact Pten-KO mice were divided into three groups (control (n = 40), plumbagin (200 ppm) (n = 20), and plumbagin (500 ppm; n = 40)). Plumbagin treatment was started when the mice were 4 weeks old, the time when hyperplasia began, and were sacrificed at 15 and 30 weeks. Development of prostate cancer in control and plumbagin-treated Pten-KO mice was evaluated by examining prostate tumor volume and weight. Plumbagin administration (200 and 500 ppm) resulted in a dose-dependent significant (P < 0.001) decrease in the urogenital apparatus (Fig. 2A, i) and prostate tumor weights (Fig. 2A, ii) at 15 weeks. We analyzed the prostate tumor volume of 3 control and 3 plumbagin-treated mice by CT/PET imaging, using tumor-specific radiopharmaceutical agent (124I-NM404) at 30 weeks (Fig. 2B, i and ii). Plumbagin treatment resulted in a significant (P < 0.001) decrease in prostate tumor volumes compared with control mice (Fig. 2B, ii). Plumbagin treated mice showed less uptake of 124I-NM404 compared with control mice which were correlated with decreased tumor volumes (Fig. 2Bi). Plumbagin treatment also resulted in a dose-dependent significant (P < 0.001) decrease in the weight of urogenital apparatus (Fig. 2C, and prostate tumor (Fig. 2C, ii) at 30 weeks. We next examined the effects of plumbagin on genitourinary apparatus of WT littermates of Pten-KO mice. In this experiment, a total of 20 WT mice (4–6 weeks old) were used. Mice were divided into two groups (control (n = 10), and plumbagin (500 ppm; n = 10)). Mice were sacrificed at 15 and 30 weeks for examining genitourinary apparatus. Results demonstrated that plumbagin treatment did not affect the weight of prostate and genitourinary apparatus of wild-type mice at 15 and 30 weeks (Fig. 2D, i–iii) compared with control. Plumbagin treatment (500 ppm) up to 30 weeks did not show any toxicity in Pten-KO mice as confirmed by histopathologic analysis of liver, lungs, kidney, and spleen (Fig. 2E, i–iv).

Plumbagin treatment inhibits progression of invasive adenocarcinoma and cystic dilation in the prostate of Pten-KO mice

To determine whether plumbagin treatment inhibits progression of PIN to invasive adenocarcinoma in Pten-KO mice, histopathologic analyses were performed in excised prostate tissues of both the control and plumbagin-treated mice as described by Kaplan-Lefko and colleagues (27). Hematoxylin and eosin (H&E)-stained mice prostate sections were microscopically examined and classified as focal PIN, diffuse PIN, small well-differentiated carcinoma, large well-differentiated carcinoma, and well-differentiated carcinoma with cystic dilation. We randomly selected 10 areas of each mouse prostate section for giving the histopathologic score. Representative images of H&E-stained sections.
of control and plumbagin-fed mice prostates are shown in Fig. 3A, i–iii and Fig. 3B, i–iii. At 15 weeks, all of the control mice prostate showed diffuse PIN and large well-differentiated carcinoma, whereas they were significantly (P < 0.001) decreased in plumbagin-treated mice in a dose-dependent manner (Fig. 3A, i–iii and 3, C–i–iii). Desmoplastic changes were observed in the prostate stroma of control mice at 15 weeks (Fig. 3A, i) which was significantly reduced in the prostate stroma of plumbagin-treated mice (Fig. 3A, ii and iii). None of the mouse either from control or plumbagin-treated groups showed well-differentiated carcinoma with cystic dilation at 15 weeks. At 30 weeks, both 200 and 500 ppm plumbagin-treated mice displayed significantly decreased incidence of diffuse PIN (P = 0.008), invasive adenocarcinoma (P < 0.001), and invasive adenocarcinoma with cystic dilation (P < 0.001) compared with control mice (Fig. 3B, i–iii, Fig. 3D, i–iii). We also performed histopathologic analysis of liver, lungs and lymph nodes of control Pten-KO mice (n = 30) and found none of the mice with prostate cancer metastasis (data not shown).

Plumbagin treatment inhibits constitutive expressions of AKT, PKCe, Stat3, and COX-2 and decreases serum IL6 level in Pten-KO mice

AKT serine/threonine kinase is one of the primary targets of the PTEN-controlled signaling pathway (28). Thus, AKT phosphorylation serves as a reliable indicator of PTEN loss. We observed an increased expression of pAKTSer473 in the prostate tissues of Pten-KO mice compared with WT as analyzed by Western blot analysis (Fig. 4A, i and ii). At 30 weeks, plumbagin treatment resulted in an inhibition of AKT phosphorylation in prostate tissues of Pten-KO mice compared with control mice (Fig. 4A, i and ii).

PKCe is a transforming oncogene and is involved in the induction and progression of various types of cancers (29, 30), including prostate (26, 31–33). We have previously reported that the expression level of PKCe and Stat3 correlates with human prostate cancer aggressiveness (31–34). We determined the effects of plumbagin on the expression of PKCe and Stat3 in excised prostate tissues of control and plumbagin-treated Pten-KO mice. Western blot analysis results demonstrated increased expression of PKCe and Stat3 in the prostate tissues of Pten-KO mice compared with the prostate of WT littermates (Fig. 4A, i and ii). Plumbagin treatment resulted in an inhibition of PKCe expression in the prostate tissues of Pten-KO mice at 30 weeks compared with the control (Fig. 4A, i and ii). We further analyzed the kinase activity of PKCe in prostate tissues of WT, control, and plumbagin-treated Pten-KO mice. Results demonstrated significant (P < 0.01) increased PKCe kinase activity in the prostate tissues of Pten-KO mice compared with WT littersmates at 15 and 30 weeks (Fig. 4B). We observed a dose-dependent decrease in PKCe activity in the prostate tissues of plumbagin-treated Pten-KO mice compared with the control (Fig. 4B). We also observed increased Stat3 phosphorylation at both Ser727 and Tyr705 residues in the prostate tissues of Pten-KO mice compared with WT littersmates (Fig. 4A, i and ii). Plumbagin treatment resulted in inhibition of both Stat3 phosphorylation at both Ser727 and Tyr705 residues compared with the control (Fig. 4A, i and ii). Accumulating evidence has suggested the link of COX-2 in prostate cancer progression (35). A recent study has shown inhibition of prostate cancer bone metastasis by COX-2 inhibitor (36). We observed increased expression of COX-2 in prostate tissues of Pten-KO mice compared with the prostate of WT littersmates (Fig. 4A, i and ii). Plumbagin treatment resulted in inhibition of COX-2 expression compared with control (Fig. 4A, i and ii). We observed an increased serum IL6 level in Pten-KO mice compared with WT littersmates, which was significantly (P < 0.01) reduced in plumbagin-treated mice (Fig. 4C). Plumbagin did not show any significant effects in other cytokines (Exotoxin, IL1α, IL2, IL4, IL5, IL9, and IL10; data not shown).

Plumbagin inhibits constitutive expression of vimentin and slug in Pten-KO mice

Vimentin and slug have been shown to be overexpressed during the EMT in most tumor types, including prostate cancer (37–39). They have also been considered as markers of EMT (40). A possibility was explored whether plumbagin administration inhibits EMT in the prostate cancer tissues of Pten-KO mice. We observed an increased expression of both vimentin and slug in the prostate tissues of Pten-KO mice compared with WT littermates (Fig. 5A, i and ii). Western blot results demonstrated inhibition in the expression of both vimentin and slug in the prostate tissues of plumbagin-treated mice (30 weeks) compared with the control (Fig 5A, i and ii). We next performed immunofluorescence analysis of vimentin and slug in prostate tissue sections of control and plumbagin-treated Pten-KO mice and observed decreased expression of both vimentin (Fig. 5B, i–iv) and slug (Fig. 5B, v–viii) in the prostate tissues of plumbagin-treated mice compared with the control.

Plumbagin inhibits CRPC in Pten-KO mice

It has been shown that Pten-KO mice develop CRPC. We next determined the effects of plumbagin administration on CRPC. In this experiment, 26 Pten-KO mice were castrated at 10 weeks and divided into two groups. One group of mice was administered plumbagin (500 ppm) in the diet, and the other group of mice was fed with the control diet. Both groups of mice were sacrificed at 50 weeks. Prostate tumor development was studied in both groups of mice by determining tumor weight and histopathologic analysis. We observed that castration of Pten-KO mice inhibited prostate tumor growth as assessed by prostate weight and histopathologic analysis (Fig. 6A and C). We observed a significant (P < 0.0001) decrease in prostate weight in plumbagin administered mice compared with control mice (Fig. 6B). Histopathologic analysis of plumbagin treated mice prostate tissues showed a significant (P < 0.01) decrease of both invasive adenocarcinoma and cystic dilation compared with untreated castrated mice (Fig. 6C).

Discussion

Because of the heterogeneous nature of prostate cancer, it is important to test the preclinical efficacy of a chemopreventive agent in various mouse models. Plumbagin is a unique plant-derived napthoquinone and has been known for its health benefits against various types of ailments, including cancer (9–22). Previous reports from our laboratory have demonstrated that plumbagin treatment inhibits (i) >90% human prostate cancer cells DU145 ectopic xenograft tumors in athymic nude mice (20), (ii) the growth and metastasis of highly aggressive human prostate cancer cells (PC-3M; ref. 21), and (iii) prostate carcinogenesis in TRAMP mice (22). A recent study has shown that the combination of plumbagin and androgen withdrawal regress mouse prostate cancer cells (PTEN-P2) derived allograft tumors in mice (41). All of these effects have been shown when
Figure 3.
Effects of plumbagin (PL) on the progression of invasive adenocarcinoma in Pten-KO mice. Histopathologic analyses of excised prostate tumor tissues at age 15 and 30 weeks. Representative photographs (final magnification proximity 20× and 200×) showing H&E staining of excised prostate tumors from control and plumbagin-treated mice at 15 (A) and 30 (B) weeks. Green and black arrows indicate PIN and invasive adenocarcinoma, respectively. Yellow arrows indicate cystic dilation along with adenocarcinoma. C–D, histopathologic analysis results of excised prostate tumors of plumbagin-treated and untreated mice at 15 and 30 weeks are summarized in bar graphs.
Plumbagin was administered intraperitoneally 1 or 2 mg/kg body weight. In this study, we report, for the first time, that plumbagin administration in the antioxidant-free powdered diet inhibits tumor development in intact and castrated Pten-KO mice. Plumbagin has also been extensively evaluated for its toxic side effects in rodents which includes diarrhea, skin rashes, hepatic (42), and reproductive toxicity (43). The LD50 for these side effects depends upon the administration method. In mice it was 8 to 65 mg/kg body weight for oral administration and 16 mg/kg body weight for intraperitoneal administration. All of these toxic side effects were dose-related and it is noteworthy that they were not observed at doses (2 mg/kg body weight i.p., and 500 nmol topical application) reported to elicit chemopreventive and therapeutic effects (17). In our findings, we observed 2,000 ppm of plumbagin in the diet is the maximum tolerated dose in mice. These mice showed normal body weight gain and exhibited no signs of toxicity in vital organs. Hsieh and colleagues have reported a single dose pharmacokinetic study of plumbagin in rats (26). In this study, rats were dosed with either 3 mg/kg i.v. or 100 mg/kg orally, demonstrating an area under the curve (AUC) of 18.76
(min g/mL) for the i.p and 272 (min g/mL) for the oral formulation (26). In our study, we detected approximately 50 ng/mL in the plasma of mice orally administered with the 2,000 ppm dose of plumbagin. These results indicate that plumbagin is moderately bioavailable, when given orally. Plumbagin was not detected in the plasma of mice fed with plumbagin (500 ppm). This may be due to low dose of plumbagin administration.

Plumbagin administration to Pten-KO showed significant ($P < 0.001$) inhibition of prostate tumor weight, volume, and invasion. Thirty weeks old Pten-KO mice illustrated well-differentiated carcinoma along with cystic dilation which was significantly ($P < 0.001$) reduced in plumbagin-administered Pten-KO mice. Our data also indicate no toxicity of plumbagin treatment (500 ppm) in the vital organs of Pten-KO mice. These results indicate the potential chemopreventive effects of plumbagin against prostate carcinogenesis. Long-term use of plumbagin has toxic side-effects, including reproductive toxicity (44). Although, plumbagin has reproductive toxicity but the old age patients suffering from primary and invasive prostate cancer may not have this issue. Our data clearly indicate that plumbagin may be used in the prevention and treatment of both primary as well as invasive prostate carcinoma.

It has been shown that plumbagin is a natural multitargeting agent which targets several signaling pathways associated with the induction and progression of prostate cancer (19–22). Accumulating evidence suggests that PKCe is an oncogene and plays an important role in the induction and progression of various types of cancers (28, 29), including prostate cancer (25, 29–32). Overexpression of PKCe is sufficient to promote conversion of prostate cancer androgen-dependent LNCaP cells to androgen-independent variant, which rapidly initiates tumor growth in vivo in both intact and castrated athymic nude mice (45). We have shown previously that the PKCe expression level correlates with the aggressiveness of human prostate cancer (30). We also have shown that genetic loss of PKCe in TRAMP mice prevents development and metastasis of prostate cancer (25). A recent study has suggested that the overexpression of PKCe in the mouse prostate epithelium promotes the development of PIN at 16 to 18 weeks.
plumbagin administration on the expressions of PKC 
vivo (32). PKC 
development is not consistent with our previous published reports where plumbagin treatment showed inhibition of PKC 
in excised prostate tissues of Pten-KO mice. Our results indicate shown that PKC 
signaling pathways in prostate cancer (46–49). We have also shown that PKC 
associates with Stat3, and this association increases with prostate cancer development and progression in human and mice (31). We have shown that constitutive inhibition of PKC 
in prostate tissues of Pten-KO mice. Our results indicate that plumbagin administration inhibits PKC 
activity in prostate tumors of Pten-KO mice. Our results are in accord with our previous published reports where plumbagin treatment showed inhibition of PKC 
and Stat3 activation in prostate cancer cells (20–22). Development of prostate cancer is not confined to the prostate epithelial cells, but also involves the tumor microenvironment (TME). Multiple signaling pathways, growth factors, and cyto-
kines exist between epithelial cells, stromal cells, and the extra-
cellular matrix to support tumor progression from the primary site to regional lymph nodes and distant metastasis (50). Our cyto-
kines array data indicate increased serum IL6 level in Pten-KO mice compared with WT littermates. These findings are in accord with previous published reports showing an association between IL6 and poor prognosis of prostate cancer (51–54). Our data showed a significant decrease in the serum IL6 level in plumbagin-administered Pten-KO mice. These findings correspond to our previous published report where plumbagin treatment inhibited the serum IL6 level in pancreatic cancer cells derived xenograft mice (19). From these results, we cannot rule out the possibility whether these molecules are the direct molecular targets of plumbagin or the indirect consequence reflecting the suppressed tumor growth in the treatment groups. However, in our previous study, we have shown in vitro that plumbagin treatment of prostate cancer cells (DU-145) for 6 hours inhibits expression of PKC 
and phosphorylation of Stat3 and AKT (20). It may be possible that these molecules are the direct molecular targets of plumbagin in prostate cancer.

EMT is a biologic process by which the normal epithelial cell acquires a mesenchymal phenotype (50). This EMT process helps cancer epithelial cell migration from primary tumor to distant metastatic sites. Several regulatory and specific biomarkers, including vimentin and slug have been shown to be modulated during EMT (50). Vimentin expression has been shown in poorly differentiated prostate cancer and bone metastases of prostate cancer (37). Other studies have shown constitutive overexpression of vimentin in highly aggressive androgen-independent prostate cancer cell lines (PC-3M), compared with androgen-independent LNCaP cell lines (39). Furthermore, it has been shown that constitutive inhibition of vimentin inhibits invasion of PC-
3M cells (54). Several other studies also supported the view that vimentin is over expressed in prostate cancer and contributes to their invasive and metastatic potentials (37–40). Our results indicate that plumbagin targets vimentin in the prostate cancer tissues of Pten-KO mice. Previous study has shown that PKC 
is involved in phosphorylation of vimentin (55). Therefore, it may be possible that plumbagin inhibits vimentin expression via inhibition of PKC 
in prostate cancer cells. Slug, a member of the Snail family of zinc-finger transcription factors (17), was 
identified as a potential oncogene in various types of cancer (18–21). The role of slug in the induction of EMT is well defined in various types of cancer, including prostate cancer (56–58). Slug induces both androgen and nonandrogen transactivation of androgen receptor signaling pathways in prostate cancer (59). Our findings indicate that plumbagin inhibits slug expression in the prostate cancer tissues of Pten-KO mice. Overall, these results indicate that plumbagin inhibits the induction of EMT in prostate cancer via inhibition of vimentin and slug expression.

In summary, our results clearly indicate that plumbagin inhibits prostate tumor development in intact and castrated Pten-KO...
mice. The antitumor potential of plumbagin in Prn-KO mice could be partly due to the inhibition of PKCe, IL6/Stat3 signaling pathways, and EMT. These results further provide evidence that plumbagin is a potential chemopreventive agent against prostate cancer. We suggest that plumbagin alone or in combination with androgen ablation should be tested in clinical trials against CRPC.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: B.B. Hafeez, J. Fischer, A. Verma
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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): B.B. Hafeez, J. Fischer, W. Zhong, A. Mustafa, M.O. Sheikhani, A. Verma

References
Epithelial-to-Mesenchymal Transition Markers

Plumbagin Inhibits Prostate Carcinogenesis in Intact and Castrated PTEN Knockout Mice via Targeting PKC ε, Stat3, and Epithelial-to-Mesenchymal Transition Markers


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