

***Phellodendron amurense* Bark Extract Prevents Progression of Prostate Tumors in Transgenic Adenocarcinoma of Mouse Prostate: Potential for Prostate Cancer Management**

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Abstract. Prostate cancer is the second leading cause of cancer-related deaths in men in Western society. Epidemiological studies suggest that a reduced risk of cancer is associated with the consumption of a phytochemical-rich diet that includes fruits and vegetables. Strategies to delay clinically significant prostate cancer will have a tremendous impact in reducing the overall incidence of prostate cancer as well as improving quality of life for elderly men. Furthermore, the long latency involved in the development of clinically significant prostate cancer provides a plethora of opportunities for its management, especially using prevention approaches. Previous studies from our laboratory show that Nexrutine^R (bark extract from *Phellodendron amurense*) prevents prostate tumor development when given prior to the development of high-grade prostatic intraepithelial neoplasia in the transgenic adenocarcinoma of mouse prostate (TRAMP) model. In this study, we investigated the effect on the progression of established tumors in the TRAMP model by administering Nexrutine^R to 28-week-old TRAMP mice. Efficacy of Nexrutine^R was determined by histopathological evaluation of the prostate. Our data indicate that Nexrutine^R inhibited progression of prostate tumors that was correlated with tissue levels of transcription factors nuclear factor kappa B, cyclic-AMP response element-binding protein and phosphorylated CREB. Moreover, Nexrutine^R intervention resulted in a significant increase in the bone mineral density of the left femur diaphysis ($p=0.009$) and prevented the

development of metastatic lesions. Nexrutine^R treatment also significantly ($p=0.005$) inhibited invasion of androgen-independent prostate cancer cells

Prostate cancer is the second leading cause of cancer-related deaths in American men, accounting for about 10% of cancer-related deaths. The American Cancer Society estimates that about 27360 men in the United States will die of prostate cancer in 2009 (1). African-American men have the highest incidence of prostate cancer in the world, whereas Asian men native to their countries who consume a low fat, high fiber diet have the lowest risk (2). Epidemiological studies suggest that a reduced risk of cancer is associated with the consumption of a phytochemical-rich diet that includes fruits and vegetables (3). Evidence suggests that clinically detectable prostate cancer does not generally manifest itself until the 6th decade of life. Such long latency involved in the development of clinically significant prostate cancer provides a plethora of opportunities for intervention, including the use of phytochemicals. In addition, since cancer arises due to deregulation of multiple signaling pathways, targeting multiple signaling pathways using a combination of agents or complex botanicals offers an added advantage of providing a synergistic or additive effect (4). These data indicate a potential for developing novel non-toxic agents from plants (phytoceuticals) for successful management of prostate cancer.

Nexrutine^R, is a commercially available herbal extract from *Phellodendron amurense* (from phellodendron, the Greek word for cork tree), which is widely used for the treatment of inflammation, gastroenteritis, abdominal pain and diarrhea (5, 6). The tree is native to Asia and has been reported to contain isoquinoline alkaloids, phenolic compounds and flavone glycosides (5, 6). Studies from our laboratory demonstrated that Nexrutine^R (i) inhibits prostate cancer cell proliferation through modulation of Akt and cyclic-AMP response element-

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binding protein-mediated signaling pathways (7); and (ii) prevents development of early-stage prostate tumors in transgenic adenocarcinoma of mouse prostate (TRAMP) model (8). Recently, we found berberine or a related compound to be an active component associated with the observed biological activity of Nexrutine^R in prostate cancer cells (9). We have also demonstrated that Nexrutine^R reduces nuclear factor kappa B transcriptional activity that is associated with the inhibition of proliferation (9). However, whether Nexrutine^R can be effective after tumors have established is not known. This is extremely important since prostate cancer patients are diagnosed with various stages of disease, therefore developing a non-toxic agent with potential for targeting both early- and late-stage disease will have tremendous translational potential (10-11). Accordingly, we tested the ability of Nexrutine^R against established tumors in TRAMP mice.

Materials and Methods

Preparation of Nexrutine^R diet. Nexrutine^R was provided by Next Pharmaceuticals, Irvine, CA, USA. Based on our published data demonstrating efficacy of Nexrutine^R, we selected 600 mg/kg Nexrutine^R for use in *in vivo* experiments (8). Pelleted diet containing 600 mg/kg Nexrutine^R was prepared at Dyets, Inc. (Bethlehem, PA, USA). Stability of Nexrutine^R in the diet pellets was evaluated monthly using thin-layer chromatographic (TLC) analysis as described elsewhere (8).

Transgenic mouse experiments. TRAMP model was developed by prostate-specific expression of SV40 large T antigen using the rat probasin promoter (12, 13). TRAMP mice develop prostate tumors with 100% frequency, in progressive stages that facilitates preclinical studies in the prevention, intervention and regression setting as demonstrated by various groups, including our own (14-21). TRAMP mice, with a pure C57BL/6 background, were obtained from Jackson Laboratories (The Jackson Laboratories, Bar Harbor, Maine, USA).

Experimental design. All mice were maintained in a climate-controlled environment with a 12-h light-dark cycle. Diet and water were supplied *ad libitum*. Administration of Nexrutine^R was initiated in 28-week-old animals, by which time they develop moderately differentiated adenocarcinoma. Control animals received diet without Nexrutine^R. Body weight changes and food consumption were measured every week during the study. Animals were euthanized after 6, 10 and 14 weeks' intervention to determine the effect of duration on the efficacy of Nexrutine^R (shown in Figure 1A). At the time of termination, all organs were collected for further analysis. At necropsy, animals were examined to determine if there were any gross organ abnormalities. Animal care and handling was conducted in accordance with established humane guidelines and protocols approved by the University of Texas Health Science Center at San Antonio's Institutional Animal Care and Use Committee.

Tumor histology. Tumors were weighed, harvested and fixed in 10% neutral buffered formalin. Tumors were paraffin embedded, sectioned, placed on slides and stained with H&E to visualize cell

nuclei and cytoplasm. Prostate lesions were scored using an established grading system for TRAMP mice as described by us before (8, 20, 21). Non-cancerous lesions were graded as 1, 2 or 3, indicating normal tissue, low grade prostatic intraepithelial neoplasia (PIN) and high grade PIN, respectively. Grades 4, 5 and 6 indicated well-differentiated, moderately differentiated and poorly-differentiated cancerous lesions, respectively. Three different sections from each tumor were analyzed for histopathological grading. The data presented are average tumor grades from all sections that were used for statistical analysis. Images were recorded using a light microscope.

DEXA imaging. Bone mineral density (BMD) was measured at the termination of the study by dual-energy X-ray absorptiometry (DEXA) in TRAMP mice receiving control and Nexrutine^R-containing diet and age-matched wild-type animals. BMD was measured essentially as described by Rahman *et al.* (22, 23) using Lunar PIXImus mouse bone densitometer (General Electric) and data analysis was carried out manually with PIXImus software. Five animals per group were used for these measurements and data presented are the average+sd BMD.

Immunohistochemical analysis. Sections from formalin-fixed, paraffin-embedded tissue blocks of prostate were cut and stained for p65, pAkt (Ser473), CREB and pCREB, cyclin D1 (Cell Signaling Danvers, MA, USA) as described elsewhere (8). The antibodies used were at a dilution of 1:100 in PBS and incubated overnight at 4°C. Immune complexes were revealed using a universal secondary antibody (100 µl for 30 minutes) followed by chromogen. Cells (both positive and negatively stained) were counted in 10 different randomly selected fields per sample to obtain quantification data.

Proliferation and terminal transferase dUTP nick end-labeling (TUNEL) staining in tumors. Proliferation was assessed using the Ki-67 (SP6) antibody (Lab Vision, Fremont, CA, USA). The secondary and tertiary antibodies were a biotinylated link and streptavidin horseradish peroxidase (HRP) (Biocare 4 plus Kit, Biocare Medical, Concord, CA, USA). Apoptosis was assessed using *in situ* the terminal transferase dUTP nick end-labeling (TUNEL) assay, with biotin-16-dUTP (Roche Applied Science, Indianapolis, IN, USA) and terminal deoxynucleotidyl transferase (TdT) according to vendor recommendations (Invitrogen, Carlsbad, CA, USA). Cells (both positive and negatively stained) were counted in 10 different randomly selected fields per sample to obtain quantification data.

Tumor invasion assay. Cell Invasion Assay (Oncogene Research Products, San Diego, CA, USA) was used to determine the direct role of Nexrutine^R in inhibiting tumor invasion as described by us before (20). This assay utilizes an invasion chamber with an 8 µm pore size polycarbonate membrane. The upper surface of this membrane is coated with a uniform layer of basement membrane matrix solution that forms an effective extracellular matrix protein barrier and prevents non-invasive cells from going through the pores. In contrast, invasive cells are able to degrade the matrix proteins that occlude the pores and allow them to pass through. The ability to invade can be quantified by labeling the invaded cells with calcein-acetomethoxy (AM) followed by fluorescence measurement. Briefly DU145 cells were plated in 24-well plate coated with basement membrane matrix and treated with either solvent control or Nexrutine^R. Cells that migrated to

the lower chamber (invading cells) through the matrigel were quantified by labeling with calcein-AM and measuring the fluorescence.

Reverse transcriptase-polymerase chain reaction (RT-PCR). RNA was prepared from LNCaP, PC-3 and DU 145 cells (obtained from American Type Culture Collection, Manassas, VA, USA) treated with Nexrutine^R or solvent control for 6 and 24-h and expression of MMP-2, MMP-3, Bcl-2, Bax and β -actin was measured by RT-PCR using One-step Access RT-PCR system (Promega Corporation Inc., Madison, WI, USA). Primers were purchased from R&D systems (R&D systems, Inc. Minneapolis, MN, USA). Data presented here are the average \pm sd of two independent experiments. Levels of MMP-2, MMP-3 and β -actin were quantified with Gene Tools software using Syngene Image station. A representative gel is shown.

Statistical analysis. For the comparison of Nexrutine^R vs. control diet modeled with diet duration of 6 vs. 10 to 14 weeks, factorial ANOVAs were performed to compare body weights at 28 weeks (when Nexrutine^R was introduced to the experimental animals), the increases in body weight during the initial 6-week duration, and the increases in body weight from 28 weeks until the animal was sacrificed. Prostate seminal vesicle complex (PSVC) weights obtained after sacrifice were also compared using factorial ANOVA. If the treatment by duration interaction term was significant, then selected Bonferroni-adjusted pairwise comparisons were performed to identify 4 specific mean differences of interest. These included comparisons of 6- vs. 10- to 14-week durations separately for the Nexrutine^R and control animals, and comparisons of Nexrutine^R vs. control separately for 6- and 10- to 14-week duration. If the interaction term was not significant, then the main effects of treatment and duration were checked for significance.

Mann-Whitney *U*-tests were performed for each intervention duration (6, 10, and 14 weeks) comparing tumor grades of control and Nexrutine^R-treated animals. Kruskal-Wallis tests were performed separately for control and Nexrutine^R-treated animals comparing tumor grades across intervention durations. If the Kruskal-Wallis tests were not significant for both treatment groups, then this was interpreted as confirming the hypothesis that no significant interaction between intervention duration and treatment group was observable for tumor grade. If the null hypothesis for the duration by treatment interaction was not rejected, then a Mann-Whitney *U*-test comparing tumor grades of control and Nexrutine^R-treated animals for all intervention durations was performed. For the Mann-Whitney *U*-tests, $p < 0.05$ was considered significant. For the Kruskal-Wallis tests, $p < 0.10$ was considered significant. Statistical analysis was performed using Stata 11.0 (StataCorp, College Station, TX, USA).

Results

Nexrutine^R intervention inhibits progression of preformed prostate tumors in TRAMP mice. As shown in Figure 1A, 28-week-old TRAMP mice were randomized into two groups of 14 animals each for control and treatment. Animals in the treatment group received Nexrutine^R 600 mg/kg diet (dose based on previous published study; (8) until the end of the experiment at 42 weeks. Five animals per group were

euthanized at the end of 6, 10 and 14 wk intervention respectively to determine the efficacy of Nexrutine^R on the progression of established tumors (one animal died from each group). Factorial ANOVA results for body weight at 28 weeks yielded a significant interaction between treatment and duration ($F=12.54$, $p=0.002$), but the only significant mean pairwise difference was for animals on the 6-week diet duration, with those for Nexrutine^R being significantly lower than the control. Despite this initial body weight difference, none of the factorial ANOVA results for body weight changes were significant, indicating non-toxicity of Nexrutine^R (data not shown). For PSVC weight measures, factorial ANOVA yielded non-significant treatment by duration interaction ($F=0.42$, $p=0.521$) and non-significant overall treatment effect ($F=0.25$, $p=0.623$), but the overall duration effect was significant ($F=9.15$, $p=0.006$); hence, mean PSVC weight for 10- to 14-week diet duration was significantly greater than that for 6-week duration, regardless of treatment (data not shown). These data indicate Nexrutine^R intervention did not affect PSVC weight significantly. As described in Materials and Methods, prostate lesions were scored using an established grading system for TRAMP mice (8, 20, 21). Tumor grade was significantly lower in Nexrutine^R-treated animals compared to control using 6- ($z=2.14$, $p=0.032$), 10- ($z=2.29$, $p=0.022$) and 14-week ($z=2.00$, $p=0.046$) interventions using Mann-Whitney *U* tests. Since Kruskal-Wallis tests indicated that tumor grade was not significantly different across weeks for controls (Chi-square=2.08, $p=0.354$) and treated animals (Chi-square=0.37, $p=0.832$), we combined the data from all intervention durations and analyzed for significance using Mann-Whitney *U*-test. These data indicate that tumor grade was significantly lower for treated compared with control animals ($z=3.90$, $p<0.001$) regardless of the duration of intervention. Tumor grades are shown in Figure 1B and representative H&E images from control and treated animals are shown in Figure 1C and D.

Nexrutine^R intervention reduces metastatic lesions in TRAMP mice. The above data indicate that oral administration of Nexrutine^R inhibited tumor development in TRAMP mice by inhibiting their progression. However, we do not know whether tumors are delayed and will appear at a later time. While 100% of animals in both groups developed tumors (varying stages), 4 out of 16 animals in the control group but none in the intervention group (0/16) developed metastatic lesions to various organs including lymph node, liver, lungs and kidney (Figure 2). These data from the control group of animals is consistent with the published results demonstrating distant metastasis to these organs in the TRAMP model (24). These results indicate that Nexrutine^R either delays or prevents metastasis to distant organs.

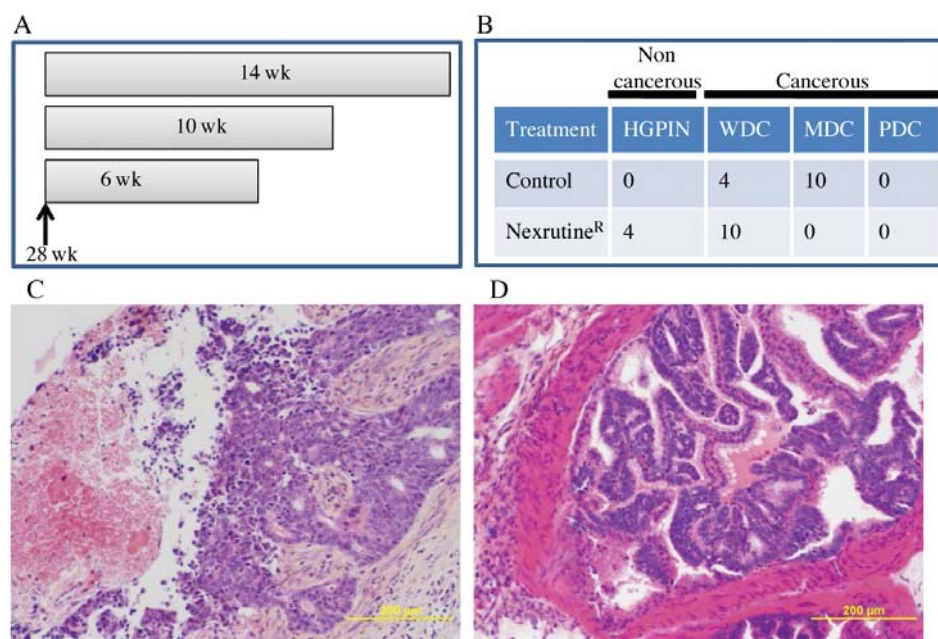


Figure 1. Nexrutine^R intervention inhibits progression of preformed prostate tumors in TRAMP mice. Twenty-eight-week-old TRAMP mice were fed control or Nexrutine^R-containing diet for a total of 14 weeks. Animals were euthanized after 6, 10 and 14 weeks intervention to determine the effect of duration on the efficacy of Nexrutine^R (panel A). Animals were observed for tumors by abdominal palpation and body weight changes throughout the study. At the time of termination and all organs were collected for histopathological evaluation. Sections of prostate tissues excised from TRAMP mice were stained with H&E. Histopathological evaluation of prostate was performed on H&E stained slides and graded. The prostatic lesions were graded as described earlier on a scale of 1 to 6. Non-cancerous lesions were graded as 1, 2 or 3 indicating normal tissue, low prostatic intraepithelial neoplasia (PIN) and high PIN respectively; grades 4, 5 and 6 indicated well-differentiated (WDC), moderately differentiated (MDC) and poorly differentiated (PDC) cancerous lesions respectively (panel B). Representative H&E images from control (C) and treatment group (D) are shown.

Nexrutine^R intervention prevents bone loss. It has been shown that bone is a preferential site of metastasis in prostate cancer patients and once the tumor metastasizes to bone, only palliative treatment options are available (25). Androgen-deprivation therapy (ADT) that is routinely used for prostate cancer management has been shown to be associated with decreased BMD with consequent increased risk of bone fractures (26-29). In addition, about 19% of men on ADT compared to 12% without ADT had fractures 5 years after the diagnosis of prostate cancer (30). We tested if Nexrutine^R can modulate BMD using DEXA in TRAMP mice compared to age-matched wild-type non-transgenic animals under similar experimental conditions. As shown in Figure 3, cancer progression was associated with a significant decrease in BMD of left femur diaphysis ($p=0.001$), left distal femur ($p=0.0001$), left proximal tibia ($p=0.005$), left tibial diaphysis ($p=0.001$), right femur diaphysis ($p=0.003$), right distal femur ($p=0.0001$), right proximal tibia ($p=0.002$), and right femur diaphysis ($p=0.001$) in TRAMP mice receiving the normal diet compared to wild-type mice. However Nexrutine^R intervention resulted in significant restoration of BMD

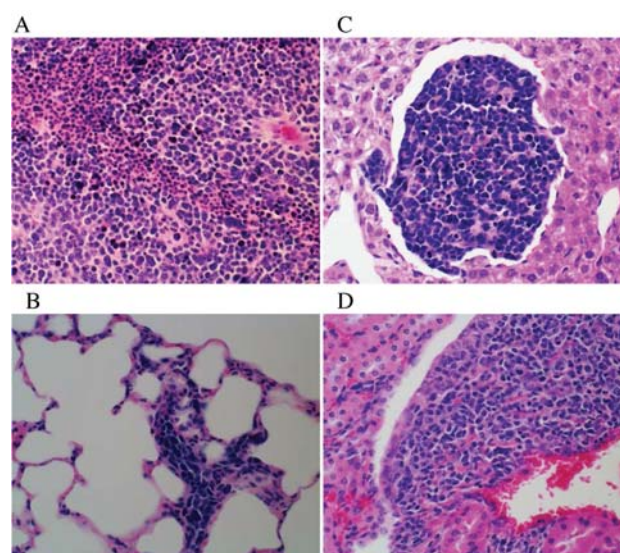


Figure 2. Nexrutine^R intervention reduces metastatic lesions in TRAMP mice. The experiment was conducted essentially as given in the Materials and Methods and examined metastatic lesions in various organs. Representative images from lymph node (A), liver (B), lungs (C) and kidney (D) are shown.

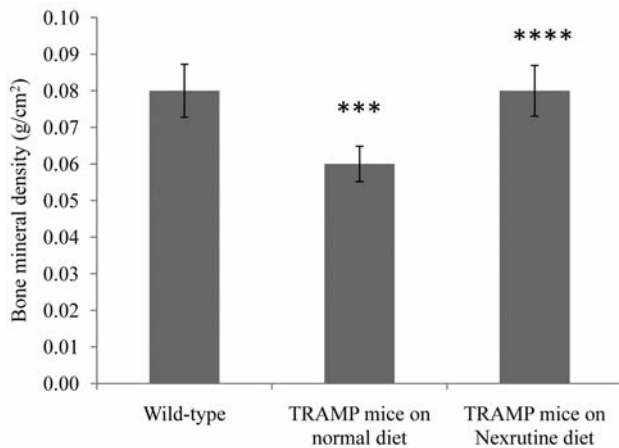


Figure 3. Nexrutine^R intervention prevents bone loss: Five animals from each of the following groups: non transgenic C57BL/6 mice, tumor bearing TRAMP mice (control) and TRAMP mice on Nexrutine^R diet were DEXA scanned using a Lunar PIXImus mouse bone densitometer (General Electric) and data analysis was carried out manually with PIXImus software. ***Compared to wild-type, and ****compared to normal diet.

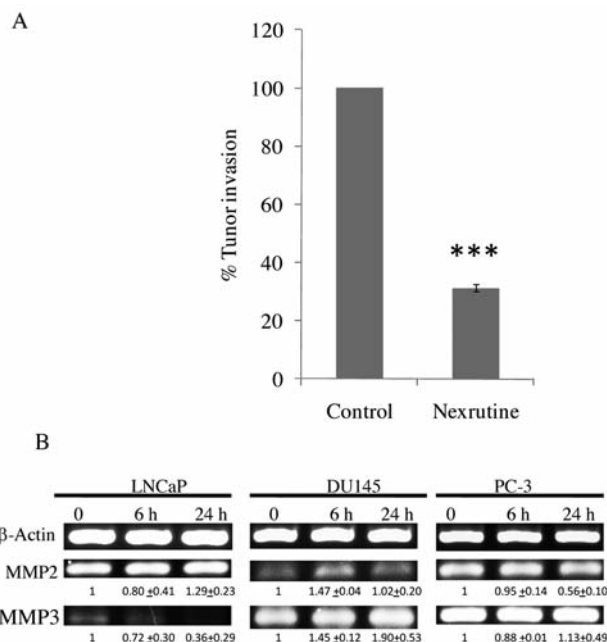


Figure 4. Nexrutine^R inhibits tumor invasion and modulates MMP2 and MMP3 expression in prostate cancer cells: A: Effect of Nexrutine^R on tumor cell invasion was determined using Cell Invasion Assay (Oncogene Research Products, San Diego, CA) as described in the Materials and Methods. The data are presented as the mean (±sd) percentage invasion with respect to the untreated control set at 100% in three independent experiments. Statistical significance ($p=0.007$ between control and treatment) was determined as described in the Materials and Methods. B: Expression of MMP-2 and MMP-3 using quantitative RT-PCR of RNA prepared from LNCaP, PC-3 and DU145 cells treated with Nexrutine^R (5 µg/ml for 6 and 24 h). A representative gel is shown.

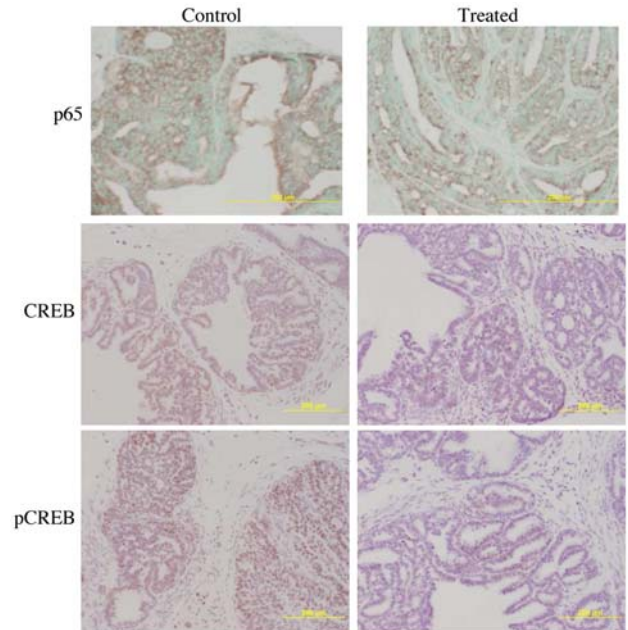


Figure 5. Immunohistochemical analysis of representative tumors or prostate tissue from control and treated TRAMP mice: Paraffin-embedded tissues sections were stained with CREB, pCREB and p65 antibodies. Negative controls were included by omitting the primary antibody.

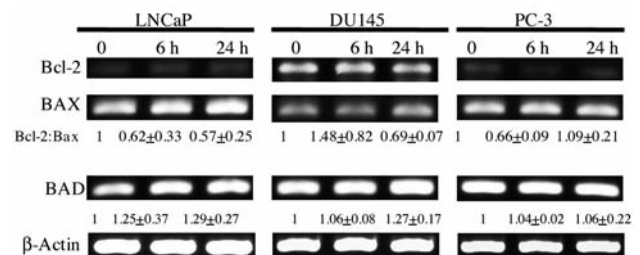


Figure 6. Expression of Bcl-2, Bax and Bad using quantitative RT-PCR of RNA prepared from LNCaP, PC-3 and DU145 cells treated with Nexrutine^R (5 µg/ml for 6 and 24 h): The ratio of Bcl-2/Bax expression was determined after normalization with β-actin internal control and is shown under the respective band.

comparable to wild-type mice only in the left femoral diaphysis ($p=0.009$). Other sites tested did not show such a statistically significant increase in BMD. These data suggest intervention with Nexrutine^R can prevent bone loss associated with cancer progression.

Nexrutine^R inhibits tumor cell invasion. One important characteristic feature of metastasis is the invasive ability of tumor cells. In order to test whether Nexrutine^R prevents tumor progression and metastasis by inhibiting tumor cell invasion, we tested the invasive potential of prostate cancer

cells using Boyden chamber invasion assay (20). Given the poor invasive ability of androgen-responsive LNCaP cells, we used androgen-independent DU145 cells to test the invasive potential of Nexrutine^R in this study. As shown in Figure 4A, 5 µg/ml Nexrutine^R inhibited the invasive capacity of DU145 cells by approximately 70% ($p=0.005$). These results suggest that Nexrutine^R can inhibit tumor cell growth not only through apoptosis, as we demonstrated earlier, but also through reducing invasion (8, 9).

Nexrutine^R modulates MMP-2 and MMP-3 expression in prostate cancer cells. Given the observation that Nexrutine^R intervention reduced metastatic lesions and inhibited tumor invasion in prostate cancer cells, we investigated changes in the expression of some of the MMPs. MMPs belong to a family of Zn-dependent proteases that degrade the structural components of extracellular matrix in addition to regulating tumor growth, apoptosis, promoting angiogenesis, inducing loss of cell adhesion facilitating invasion and metastasis (31, 32). Particularly, expression of MMP-2 in human prostate cancer tissue has been shown to be correlated with invasion and metastasis (33, 34). Additionally, levels of MMP-3 were significantly higher in prostate cancer patients with metastasis compared to controls and patients without metastasis (35). We investigated whether the observed inhibitory effect of Nexrutine^R on invasion is due to suppression of MMP-2 and -3 using RT-PCR. As shown in Figure 4B, although 5 µg/ml Nexrutine^R treatment did not affect the levels of MMP-2 or -3 in DU145 cells, it reduced MMP-2 in PC-3 cells derived from a patient with bone metastasis patient and MMP-3 in LNCaP cells. These data suggest antimetastatic activity of Nexrutine^R observed may occur independently of MMP-2 and MMP-3 in DU145 cells and warrant studies to delineate the precise mechanism of action including the role of other MMPs.

Mechanism of action of Nexrutine^R in vivo. Previously we demonstrated that prevention of tumor development in TRAMP mice with Nexrutine^R is associated with reduced expression of pAkt, pCREB and cyclin D1 and p65 (8-9). However, it is not known whether the observed inhibition of established tumors in TRAMP mice also occurs through modulation of these signaling molecules *in vivo*. Here, we tested the expression of pAkt, CREB, pCREB, cyclin D1 and p65 in the prostate from TRAMP mice administered Nexrutine^R and age-matched control TRAMP mice receiving normal diet using immunohistochemistry. As shown in Figure 5, prostate from TRAMP mice receiving the normal diet showed increased expression of p65, CREB and pCREB, consistent with published results (8-9). However, prostate from the Nexrutine^R intervention group showed consistently reduced expression of p65, CREB and pCREB. In addition, we did not observe any significant differences in the

expression of pAkt or cyclin D1 in the prostate from these groups (data not shown). It is well established that one mechanism through which Akt regulates tumor cell growth and survival is through inactivation of pro-apoptotic molecules (36). Similarly cyclin D1 regulates tumor cell proliferation through modulation of cell cycle progression (37). This prompted us to investigate the effect of Nexrutine^R on proliferation and apoptosis *in vivo* under these experimental conditions.

Nexrutine^R intervention has no effect on proliferation and apoptosis of preformed prostate tumors. Previously, we showed that Nexrutine^R intervention prevented tumor development when administered prior to their development through modulation of proliferation and apoptosis *in vivo* associated with reduction in the levels of pAkt and cyclin D1 (8). Here we investigated if intervention with Nexrutine^R inhibited tumor cell proliferation and induction of apoptosis *in vivo* using Ki-67, as a proliferation marker, and TUNEL staining to determine apoptosis. Immunohistochemical analyses show that prostate from TRAMP mice receiving the control diet were highly positive for Ki-67 immunostaining and therefore highly proliferative. However, we did not observe any significant difference in the number of Ki-67- or TUNEL-positive cells in response to Nexrutine^R intervention (data not shown).

Nexrutine^R modulates Bcl-2/Bax ratio in prostate cancer cells. Previously we have shown that Nexrutine^R induces apoptosis in androgen-responsive cells through a decrease in the ratio of Bcl-2/Bax (7). To investigate the mechanistic basis of Nexrutine^R-induced apoptosis in androgen-independent cells, we examined the levels of Bcl-2 and Bax by quantitative RT-PCR in PC-3 and DU145 cells. Beta-actin (β -actin) was used as an internal control for RT-PCR. Results presented in Figure 6 show the amplification products of *Bcl-2*, *Bax*, and *Bad* along with β -actin. Consistent with our published results, treatment of LNCaP cells showed a decrease in the ratio of Bcl-2/Bax within 6 h (7). Although similar levels of inhibition were observed in PC-3 cells, this returned to control levels by 24 h. In contrast, this ratio decreased only after 24 h treatment in DU145 cells. Increased Bcl2/Bax ratio favors the formation of antiapoptotic heterodimerization of Bcl-2/Bax and homodimerization between Bcl-2/Bcl-2, leading to inhibition of apoptosis. On the other hand, a decrease in the ratio of Bcl-2/Bax favors the formation of Bax/Bax homodimerization, leading to induction of apoptosis. Although differences exist in the kinetics of Bcl-2/Bax modulation, Nexrutine^R-induced apoptosis in prostate cancer cells involves modulation of the Bcl-2/Bax ratio. It should be mentioned that although Nexrutine^R inhibits growth of prostate cancer cells through induction of apoptosis, other mechanisms including autophagy at the cellular/molecular level *in vivo* cannot be ruled out.

Discussion

Although prostate cancer is the second leading cause of cancer-related deaths in men, the 5-year survival rate is almost 100% when the disease is confined to the prostate gland. However, 5-year survival for patients diagnosed with metastatic prostate cancer is less than 32%. Approximately 50-80% of all the patients diagnosed with prostate cancer are predicted to have metastasis to bone at the time of their death. ADT which is a standard treatment for metastatic disease produces skeletal complications including bone loss (29). Such skeletal complications lead to a poor quality of life. Currently there are no effective non-toxic strategies available to prevent bone loss that occurs either during treatment or due to metastasis. Although bisphosphonates have been shown to be effective in reducing skeletal complications, their prolonged use is associated with renal toxicity and osteonecrosis (30, 31). In addition, most patients die from metastatic spread of tumor cells to other sites, typically to bone. Such bone metastatic prostate cancer causes tremendous morbidity including pain, pathological fractures and other problems. Therefore novel strategies or compounds that can prevent such metastatic spread are urgently needed. Even if such strategies do not completely prevent the metastatic spread, delaying occurrence of such lesions will significantly improve the quality of life in this patient population. In the present study, we showed that a non-toxic natural herbal supplement inhibits (i) progression of moderately differentiated prostate tumors in TRAMP mice; (ii) development of metastatic lesions and bone loss; and (iii) levels of p65 and pCREB in the prostate. To the best of our knowledge, this is the first report that shows the potential of Nexrutine^R in preventing bone loss associated with cancer progression and metastasis. However the precise mechanism associated with restoration of BMD is not known. Data presented in this manuscript showing reduced expression of p65, CREB and pCREB are consistent with our published results showing inhibition of early-stage tumor development in the TRAMP model through modulation of CREB, pCREB and p65. However in that study, tumor growth inhibition was associated with reduction in the levels of pAkt and cyclin D1 unlike this study (8, 9). A potential reason for a lack of effect on pAkt and cyclin D1 expression in this study could be that Nexrutine^R may not prevent late-stage tumor development involving Akt and cyclin D1. Our results also indicate that apoptosis induction may not be the primary mechanism involved in inhibition of tumor development *in vivo*; however it may inhibit proliferation of prostate cancer cells through induction of apoptosis through modulation of the Bcl-2/Bax ratio. Alternatively, since we measured induction of apoptosis as an end point (at the time of termination of

the study), given the dynamic and complex nature of apoptosis, more detailed studies during prostate cancer progression as a function of age are required to address the role of apoptosis in the observed tumor growth inhibition. In addition, it is possible that Nexrutine^R may prevent late-stage tumor development through autophagy (38). Taken together, we interpret our findings to suggest that Nexrutine^R may inhibit early-stage tumor development through modulation of proliferation and apoptosis (Akt/cyclin D1) and late-stage tumor development through modulation of tumor invasion (NF- κ B/CREB). Recent results from our laboratory using normal and high Gleason grade ($\geq 8/10$) prostate tissue demonstrated that tumor cells consistently showed high intensity staining for cyclic-AMP response element-binding protein (CREB and pCREB) compared to normal epithelium (39). Subsequent studies from Dr. Chung's group showed positive association of CREB and pCREB expression with poorly differentiated cancer and bone metastatic tissue (40). In addition, we showed that Nexrutine^R inhibits invasive ability of androgen-independent prostate cancer cells. Since transcription factor NF- κ B and CREB can regulate MMPs, we speculate that Nexrutine^R inhibits tumor invasion through modulation of NF- κ B/CREB-mediated activation of MMPs. In addition, it is possible that Nexrutine^R-mediated reduction in the levels of proinflammatory cytokines and chemokines may play a role in modulating NF- κ B signaling in the observed inhibition of bone loss. A limitation of this study is the use of TRAMP mice in a C57BL/6 background since it has been shown that these mice develop bone metastatic lesions only in FVB background (24). Therefore the observed bone loss may not be related to bone metastasis directly and should be tested in a preclinical model that develops bone metastasis. Nonetheless, since ADT is associated with bone loss, use of Nexrutine^R in conjunction with ADT may decrease such effects. Studies are in progress to delineate such mechanisms in prostate cancer metastasis. In conclusion, these results imply the potential of Nexrutine^R for controlling tumor metastasis based on the observation of its inhibitory effect on invasion of cancer cells *in vitro* and inhibition of tumor metastasis *in vivo*. Therefore, this may be a potential secondary cancer prevention agent to be used against prostate cancer metastasis. In addition studies are warranted to test its utility in bone-related diseases including hormone therapy-induced bone loss associated with breast cancer.

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