A Synthetic 15-mer Peptide (PCK3145) Derived from Prostate Secretory Protein Can Reduce Tumor Growth, Experimental Skeletal Metastases, and Malignancy-Associated Hypercalcemia

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ABSTRACT

In previous studies, we have shown that prostate secretory protein (PSP-94) can reduce prostate cancer growth in vivo. In the current study, we identified the amino acid sequence of PSP-94 that is required for eliciting this response. For these studies, we used rat prostate cancer Mat Ly Lu cells overexpressing parathyroid hormone-related protein (PTHrP), which is the main pathogenetic factor responsible for hypercalcemia of malignancy. Synthetic peptides corresponding to amino acids 7–21 (PCK721), 31–45 (PCK3145), and 76–94 (PCK7694) of PSP-94 were synthesized. Only PCK3145 showed a significant reduction in tumor cell proliferation. For in vivo studies, syngenic male Copenhagen rats were inoculated s.c. with Mat Ly Lu cells overexpressing PTHrP into the right flank or into the left ventricle via intracardiac injection, which results in experimental metastases to the lumbar vertebrae causing hind-limb paralysis. Animals were infused with different doses (1, 10, and 100 μg/kg/day) of peptides for 15 days, and the effect of these treatments on tumor volume, skeletal metastases, or development of hind-limb paralysis was determined. Treatment with PCK3145 resulted in a dose-dependent decrease in tumor volume and delay in the development of skeletal metastases. Bone histomorphometry showed that after intracardiac inoculation of tumor cells, the highest dose of PCK3145 (100 μg/kg/day) resulted in reduction of skeletal tumor burden, which delayed the development of hind-limb paralysis. Treatment with PCK3145 led to reduction of plasma calcium and PTHrP levels and a significant decrease in PTHrP levels in the primary tumors and in vertebrae of experimental animals. These effects of PCK3145 were due to its ability to promote tumor cell apoptosis. Collectively, the results of these studies have demonstrated the ability of a small peptide derived from PSP-94 to reduce tumor volume and experimental skeletal metastases—results that will be highly beneficial in the continued development of this peptide as a novel therapeutic agent for patients with hormone refractory, late-stage prostate cancer.

INTRODUCTION

Adenocarcinoma of the prostate remains one of the most commonly diagnosed cancers in men and is the second leading cause of cancer death following lung cancer (1). Due to advances in detection and screening of prostate cancer, an increased number of men are being diagnosed with prostate cancer (2). Despite these advances, prostate cancer-associated mortality has remained unchanged due to the limited therapeutic strategies available. Surgery and radiotherapy still remain the most common approaches for early-stage organ-confined prostate cancer (3). However, limited success has been obtained when treating late-stage hormone-independent prostate cancer (4). This is due to the fact that increased production of several growth factors and proteases by tumor cells and the surrounding stroma leads to increased tumor cell proliferation, decreased apoptosis, and increased angiogenesis as well as inducing an increased propensity for osteoblastic skeletal metastases, which contributes to the high rate of morbidity and mortality associated with prostate cancer (5–12). Additionally, a number of patients with late-stage prostate cancer present with symptoms of increased plasma calcium levels due to the production of PTHrP by tumor cells (13). PTHrP has been identified as the major factor responsible for hypercalcemia of malignancy in several cancers including prostate carcinoma (14–16).

Prostate secretory protein 94 (PSP-94) was initially discovered as a hormone that was able to reduce the levels of circulating follicle-stimulating hormone (17). PSP-94 has since been shown to be one of the three predominant proteins secreted by the prostate gland along with prostate-specific antigen and prostatic acid phosphatase (18). Since its discovery, it has been shown that PSP-94 is produced by organs other than the prostate, especially female reproductive tissues such as the breast and ovaries (19). However, the expression of PSP-94 is highest in the prostate gland compared with other tissues (19). In addition to the 94-amino acid isoform, a less common isoform of PSP exists, which is composed of 57 amino acids (PSP-57; Ref. 20).

The expression of PSP-94 has been shown to be dependent on the stage of prostate cancer. The level of expression of PSP-94 in the normal prostate is high but progressively decreases as the prostate cancer advances from an early, low invasive, androgen-dependent state to a late, highly invasive, androgen-independent state, with a complete loss of PSP-94 expression in highly advanced prostate cancer (21, 22). This differential expression and the added advantage that the expression of PSP-94 is androgen independent compared with prostate-specific antigen and prostatic acid phosphatase could prove to be beneficial as a prognostic marker for prostate cancer patients (23).

We have previously demonstrated the ability of this naturally occurring protein (PSP-94) to reduce tumor growth and hypercalcemia of malignancy in a syngenic model of rat prostate cancer (24). In the present study, we have identified the amino acid sequence of PSP-94 that can elicit these biological responses. For these studies, we have used our well-established syngenic in vivo model of prostate cancer using the rat prostate cancer cell line Dunning R3327 Mat Ly Lu transfected with the full-length cDNA encoding PTHrP (8). Injection of these tumor cells s.c. into the right flank of male Copenhagen rats results in the development of primary tumors, whereas intracardiac (i.c.) injection into the left ventricle routinely results in experimental skeletal metastases to the lumbar vertebrae as evident by the development of hind-limb paralysis (7, 8, 25–28). Using these in vivo models, we have evaluated the effects of different peptides derived from PSP-94 to reduce tumor volume, delay experimental skeletal metastases, and reduce plasma calcium, plasma PTHrP, and tumoral PTHrP production.

MATERIALS AND METHODS

Materials. PCK721, PCK3145, and PCK7694 were gifts from Procyon BioPharma, Inc. (Montreal, Quebec, Canada).
Cells and Cell Culture. The Dunning R3327 Mat Ly Lu cell line was obtained from Dr. J. T. Isaacs (John Hopkins School of Medicine, Baltimore, MD) and transfected with full-length cDNA encoding rPTHrP as described previously (8). One of the three well-characterized monoclonal cell lines Mat Ly Lu cells overexpressing parathyroid hormone-related protein 8 (Mat Ly Lu-PTHrP-8) was used throughout the course of these studies according to previously established methods of culture of these experimental cells (8).

Morphological analysis of control and experimental Mat Ly Lu-PTHrP cells treated with PCK3145 was carried out by plating 5 × 10⁴ cells/well in 6-well plates (Falcon Plastics, Oxnard, CA) in the presence of 10% fetal bovine serum. Cells were examined daily for any change in their morphology and photographed (29).

For growth curves, Mat Ly Lu-PTHrP cells were plated in 6-well plates (Falcon Plastics) at seeding densities of 5 × 10⁴ cells/well. For 5 days, cells from triplicate wells were cultured in the presence of different doses of PCK721, PCK3145, and PCK7694 (1, 10, or 100 μg/ml), trypsinized, resuspended, and counted in a model Z Coulter counter (Coulter Electronics, Beds, United Kingdom). Medium was changed every 2 days.

Animal Protocols. Inbred male Copenhagen rats weighing 200–250 g were obtained from Harlan Sprague Dawley (Indianapolis, IN). Before inoculation, Mat Ly Lu-PTHrP tumor cells growing in serum-containing medium were washed with Hank’s buffer, trypsinized, and collected by centrifugation at 1500 rpm for 5 min. Cell pellets (5 × 10⁵ cells) were resuspended in 100 μl of saline and injected using 1-ml insulin syringes into the right flank of male Copenhagen rats. Starting on the day of tumor cell inoculation, experimental animals were treated with different doses (1, 10, or 100 μg/kg/day) of PCK721, PCK3145, and PCK7694 (1, 10, or 100 μg/ml) of PTO-94 derived peptides via s.c. injections for 15 consecutive days. Control animals received PBS alone as vehicle control. All animals were numbered, kept separately, and monitored daily for the development of tumors. The tumor mass was measured in two dimensions by calipers, and tumor volume was calculated according to the equation (l × w × h) 1/2 (where l is length and w is width). All control and experimental animals were weighed every alternate day to determine any adverse effects of PCK3145. Both control and experimental animals were sacrificed on day 16 post tumor cell inoculation, and their tumors were removed and weighed. Additionally, these tumors were used for histological analysis as described below. Blood from all control and experimental animals was collected on day 16 for determination of plasma Ca²⁺ and PTHrP levels. Plasma Ca²⁺ was measured by atomic absorption spectrometry (model 703; Perkin-Elmer, Norwalk, CT), and plasma PTHrP levels were determined by a PTHrP radioimmunoassay (Nichols Institute Diagnostics, San Juan Capistrano, CA) according to manufacturer’s instructions. Alternatively, cell pellets (10 × 10⁶ cells) were resuspended in 100 μl of saline and injected using 1-ml insulin syringes into the left ventricle of rats anesthetized with ketamine/xylazine mixture. Animals were divided into control groups that received vehicle alone (PBS) and experimental groups that were infused via i.p. injections of a single i.v. injection of various doses (1, 10, or 100 mg/kg) of PCK3145.

For determining the level of expression of PTHrP by skeletal metastases, the sections were incubated in 1% normal goat sera (Vector Laboratories, Inc.) for 30 min at room temperature and then subsequently developed with Fast Red TR/Naphthol AS-MX phosphate (Sigma-Aldrich, Oakville, Ontario, Canada) containing 1 mM levamisole for 10–15 min. The slides were then counterstained with hematoxylin (Fisher Scientific, Ltd., Nepean, Ontario, Canada) and mounted with Kaiser’s glycerol jelly. All sections were washed three times, 10 min each, with Tris buffer (pH 7.6) after each step.

Vertebral columns were fixed and decalcified for a period of 3 weeks. Vertebral column samples were dehydrated in xylene, and rehydrated through a graded alcohol series (100–70%). For determining the level of expression of PTHrP by skeletal metastases, the sections were incubated in 1% normal goat sera (Vector Laboratories, Inc.) for 30 min at room temperature before treatment with the primary antibody (polyclonal antiserum against PTHrP (1–34) from rabbit) at 1:200 dilution overnight at 4°C. Biotinylated goat antirabbit IgG (Vector Laboratories, Inc.) was used as the secondary antibody at 1:200 for 30 min at room temperature, and subsequently developed with Fast Red TR/Naphthol AS-MX phosphate (Sigma-Aldrich, Oakville, Ontario, Canada) containing 1 mM levamisole for 10–15 min. The slides were then counterstained with hematoxylin (Fisher Scientific, Ltd., Nepean, Ontario, Canada) and mounted with Kaiser’s glycerol jelly. All sections were washed three times, 10 min each, with Tris buffer (pH 7.6) after each step.

Histological Analysis. Paraffin-embedded tumor samples were cut into 5-μm-thick sections for immunohistochemical analysis. Immunohistochemical staining for PTHrP was performed using an antibody directed against PTHrP (1–34) as previously described using the avidin-biotin-peroxidase complex method (30, 31). In brief, the sections were dewaxed in xylene and rehydrated through a series of ethanol to water gradients. The sections were incubated in 1% normal goat sera (Vector Laboratories, Inc., Burlingame, CA) for 30 min at room temperature before treatment with the primary antibody [polyclonal antiserum against PTHrP (1–34) from rabbit] at 1:200 dilution overnight at 4°C. Biotinylated goat antirabbit IgG (Vector Laboratories, Inc.) was used as the secondary antibody at 1:200 for 30 min at room temperature. The slides were then treated with Vectastain ABC-AP kit (Vector Laboratories, Inc.) and then incubated 1:200 for 30 min at room temperature, and subsequently developed with Fast Red TR/Naphthol AS-MX phosphate (Sigma-Aldrich, Oakville, Ontario, Canada) containing 1 mM levamisole for 10–15 min. The slides were then counterstained with hematoxylin (Fisher Scientific, Ltd., Nepean, Ontario, Canada) and mounted with Kaiser’s glycerol jelly. All sections were washed three times, 10 min each, with Tris buffer (pH 7.6) after each step.

Vertebral columns were fixed and decalcified for a period of 3 weeks. Vertebral column samples were dehydrated in xylene, and rehydrated through a graded alcohol series (100–70%). Tissues were incubated with proteinase K for 30 min at 37°C fixed, blocked, and permeabilized. Apoptotic cells were detected by TUNEL assay in situ cell death detection kit (Roche Molecular Biochemicals, Laval, Quebec, Canada) according to the manufacturer's instruction. Positive TUNEL staining was visualized by fluorescence microscopy. In other experiments after TUNEL assay, tissue sections were counterstained with Hoechst 33258 (Sigma-Aldrich). Hoechst staining was added to tissues at a final concentration of 24 μg/ml in PBS and incubated for 15 min at room temperature. Tissue sections were washed and visualized by fluorescence microscopy using a blue screen. All results of immunohistochemistry and TUNEL assay were evaluated and interpreted by two independent examiners.

Computer-Assisted Image Analysis. Computer-assisted image analysis was carried out to quantify PTHrP immunostaining and determination of percentage of ratio of tumor volume to bone volume in the vertebral sections (31). In brief, images of stained sections were photographed with a Leica digital camera and processed using BioQuant image analysis software, version 6.50.10 (BioQuant Image Analysis Corporation, Nashville, TN). The threshold was set by determining the positive staining of control sections and was used to automatically analyze all recorded images of all samples that were stained in the same session under identical conditions. The area of stained regions was calculated automatically by the software in each microscopic field. Pixel counts of the immunoreaction product were calculated automatically and were given as total density of the integrated immunostaining over a given area.

For TUNEL assay quantification, images of stained tumor sections were taken with a Sony digital camera and processed using Northern Eclipse image analysis software, version 5.0 (Empix Imaging Inc., Mississauga, Ontario, Canada) as described previously (31).

Statistical Analysis. Results are expressed as the mean ± SE of at least triplicate determinations, and statistical comparisons are based on the Student’s t test or ANOVA. P < 0.05 was considered to be significant. Regression analysis was used to determine the effect of PCK3145 on Mat Ly Lu-PTHrP cell growth.
RESULTS

Effect of PCK721, PCK3145, or PCK7694 on Mat Ly Lu-PTHrP Cell Growth and Morphology. Mat Ly Lu-PTHrP cells were seeded in 6-well plates and treated either with different doses (1–100 μg/ml) of PCK721, PCK3145, or PCK7694 or with vehicle alone for up to 5 days. All doses of PCK721 and PCK7694 had no effect in altering cell growth of Mat Ly Lu-PTHrP cells (data not shown). Treatment with PCK3145 resulted in a dose-dependent decrease in cell growth with the highest dose (100 μg/ml) exhibiting a significant reduction in cell number compared with control Mat Ly Lu-PTHrP cells treated with vehicle alone (Fig. 1). In addition, Mat Ly Lu-PTHrP cells treated with 100 μg/ml PCK3145 changed their morphology from a more spindle-like appearance to a more circular and condensed shape (data not shown).

Effect of PCK3145 on Mat Ly Lu-PTHrP Tumor Growth and Weight. Male Copenhagen rats inoculated with Mat Ly Lu-PTHrP cells via s.c. route of injection into the right flank were infused via s.c. route, below the site of tumor cell inoculation, with different doses (1–100 μg/kg/day) of the PSP-94-derived peptides starting from the day of tumor cell inoculation and lasting for 15 days. The effect of these peptides on reducing tumor growth was evaluated by daily determination of tumor volume, and comparison was made with control tumor-bearing animals receiving vehicle alone. A progressive decrease in tumor size was seen in control animals throughout the duration of these studies. Both PCK721 and PCK7694 failed to exhibit any change in tumor volume (data not shown). In contrast, experimental animals receiving PCK3145 showed a significant dose-dependent reduction in tumor volume throughout the course of these studies (Fig. 2A). These effects of PCK3145 were similar to those of PSP-94 as previously reported (24).

To determine the effect of PCK3145 on skeletal tumor burden, control and experimental animals were sacrificed at day 10 post tumor cell inoculation when these animals do not exhibit any sign of hind-limb paralysis.

Effect of PCK3145 on the Development of Experimental Skeletal Metastases. Male Copenhagen rats were inoculated with Mat Ly Lu-PTHrP cells into the left ventricle via i.c. injections. Starting on the day of tumor cell inoculation (day 0), experimental animals received different doses of PCK3145 (1–100 μg/kg/day) via i.p. route. To evaluate the effect of PCK3145 on delaying the development of experimental skeletal metastases, animals were evaluated daily by monitoring of the animals for the development of hind-limb paralysis. All (100%) control animals inoculated with Mat Ly Lu-PTHrP cells and receiving vehicle alone developed hind-limb paralysis by day 13. Treatment with 1 and 10 μg/kg/day PCK3145 had no statistically significant effect on delaying the time of developing hind-limb paralysis (data not shown). In contrast, treatment with 100 μg/kg/day PCK3145 and 10 μg/kg/day PSP-94 resulted in a statistically significant delay in the number of animals developing hind-limb paralysis. The percentage of the total number of animals not developing hind-limb paralysis at different days is shown in Fig. 3.

A

Fig. 1. Effect of PCK3145 on Mat Ly Lu-PTHrP cell growth. Mat Ly Lu-PTHrP cells were seeded at a density of 5 × 10^5 cells/well in 6-well plates, treated with different doses (1–100 μg/ml) of PCK3145, and trypsinized and counted using a Coulter counter as described in “Materials and Methods.” Change in cell number after treatment with 1–100 μg/ml PCK3145 for 5 days is shown. Results represent the mean of three different experiments in which significant differences from control (CTL) cells are represented by * (P < 0.05).

B

Fig. 2. Effect of PCK3145 on Mat Ly Lu-PTHrP tumor volume and weight. Male Copenhagen rats received injections s.c. in the right flank with 5 × 10^5 Mat Ly Lu-PTHrP cells. Starting from the day of tumor cell inoculation, animals were infused daily with different doses of PCK3145 for 15 consecutive days as described in “Materials and Methods.” A, tumor volume was measured at timed intervals, and comparison was made with that of tumor-bearing animals receiving vehicle alone as control (CTL). B, at day 16 post tumor cell inoculation, control and experimental animals were sacrificed, and their tumors were removed and weighed. Results shown represent the mean ± SE of five animals in each group in three different experiments. Significant differences from control tumor-bearing animals receiving vehicle alone are represented by * (P < 0.05).
Limb paralysis. Vertebræ from control and experimental animals were removed, decalcified, and subjected to bone histomorphometric analysis. A significant decrease in the ratio of tumor volume to bone volume was observed in experimental animals receiving 100 μg/kg PCK3145 (Fig. 4). In the vertebrae of control animals, clear evidence of both trabecular and cortical bone destruction was seen, which resulted in the compression of the spinal cord to cause hind-limb paralysis at later time points. In contrast, these effects were significantly less in experimental animals receiving PCK3145. These vertebrae were also evaluated for the level of PTHrP expression by immunohistochemical analysis using an antibody directed against PTHrP (1–34). Control vertebrae from animals receiving vehicle alone showed strong staining for PTHrP that was significantly lower in Mat Ly Lu-PTHrP cells present in the vertebral column of animals treated with 100 μg/kg PCK3145 (Fig. 5).

**Effect of PCK3145 on Plasma PTHrP and Calcium Levels and Tumoral PTHrP Production.** To determine the effect of PCK3145 on plasma PTHrP and calcium levels, animals inoculated with Mat Ly Lu-PTHrP cells via s.c. route were sacrificed at the end of these studies, plasma was collected, and PTHrP levels were analyzed using a radioimmunoassay. Comparison was made between plasma collected from normal, non-tumor-bearing animals, control tumor-bearing animals receiving vehicle alone, and experimental animals receiving different doses of PCK3145 (1–100 μg/kg/day). Normal non-tumor-bearing animals showed basal levels of plasma PTHrP, whereas animals inoculated with Mat Ly Lu-PTHrP cells and receiving vehicle alone showed marked elevated levels of immunoreactive plasma PTHrP levels. Treatment of tumor-bearing animals with 10 and 100 μg/kg/day PCK3145 resulted in a dose-dependent decrease in plasma PTHrP levels (Fig. 6A). Analysis of plasma collected from normal non-tumor-bearing animals and tumor-bearing animals receiving vehicle alone revealed the hypercalcemic nature of control tumor-bearing animals at the time of sacrifice on day 16 post tumor cell inoculation due to a marked increase in plasma calcium levels. In contrast, experimental groups of animals receiving 10 and 100 μg/kg/day PCK3145 exhibited a significant reduction in their plasma calcium levels. Treatment with 100 μg/kg/day PCK3145 resulted in near normalization of plasma calcium levels of these experimental animals (Fig. 6B).

Tumors from control group treated with vehicle alone and experimental group treated with the highest dose of PCK3145 (100 μg/kg/day) were excised and analyzed for tumoral PTHrP production by immunohistochemical analysis. Intense PTHrP staining of tumor cells from control groups of animals receiving vehicle alone was observed (Fig. 7). In contrast, a dose-dependent decrease in PTHrP immunostaining was observed in experimental tumors from animals receiving different doses of PCK3145 (data not shown). These effects were most pronounced in tumors from animals receiving 100 μg/kg PCK3145 (Fig. 7). No significant levels of PTHrP staining were observed when these histological sections were incubated with anti-PTHrP antibody, which was pre-absorbed with PTHrP peptide (1–34) (Fig. 7).

**Effect of PCK3145 on Mat Ly Lu-PTHrP Tumor Cell Apoptosis in Vivo.** To investigate an underlying molecular mechanism of action of PCK3145 in reducing tumor growth, Mat Ly Lu-PTHrP tumors were analyzed using TUNEL assay, which can serve as a marker for apoptosis. Tumor sections from animals treated with PCK3145 (100 μg/kg/day) were significantly more TUNEL positive compared with vehicle-treated control tumors (Fig. 8). Counterstaining with H&E revealed marked TUNEL positive apoptotic changes in tumor sections from control (CTL) and experimental animals receiving PCK3145 (P < 0.05).

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**Fig. 3.** Effect of PSP-94 and PCK3145 on experimental skeletal metastases. Male Copenhagen rats were inoculated via i.c. route into the left ventricle with 10 × 10⁶ Mat Ly Lu-PTHrP cells. From the day of tumor cell inoculation, animals were infused daily with vehicle alone as control (CTL) or different doses (1–100 μg/kg/day) of PSP-94 and PCK3145 as described in “Materials and Methods.” The effect of these treatments on the development of hind-limb paralysis was evaluated daily. Percentages of animals nonparalyzed at different time points is shown. Results represent the mean ± SE of five animals in each group in three different experiments. Significant differences in percentage of nonparalyzed animals from control tumor-bearing animals receiving vehicle alone are represented by * (P < 0.05). --, CTL; ---, PSP-94 10.0 μg/kg/day; ----, PCK3145 100.0 μg/kg/day.

**Fig. 4.** Effect of PCK3145 treatment on the degree of skeletal metastases. Male Copenhagen rats were inoculated with 10 × 10⁶ Mat Ly Lu-PTHrP cells via i.c. route into the left ventricle. Animals were infused with vehicle alone or the highest dose of PCK3145 (100 μg/kg/day) starting from the day of tumor cell inoculation for up to 10 days as described in “Materials and Methods.” All animals were sacrificed on day 10, and their vertebrae were removed, decalcified, paraffin embedded, and sectioned. Histological sections of vertebrae from animals receiving vehicle alone or PCK3145 were stained with H&E as described in “Materials and Methods.” A representative photomicrograph of three such experiments is shown in the top panel. Magnification, ×200. The ratio of tumor volume to bone volume (TV/BV) was determined as described in “Materials and Methods” and is shown in the bottom panel. Results represent the mean ± SE of five animals in each group in three different experiments. Significant differences in the ratio of tumor volume to bone volume from control (CTL) tumor-bearing animals receiving vehicle alone are represented by * (P < 0.05).
Fig. 5. Effect of PCK3145 treatment on PTHrP production in experimental skeletal metastases. Male Copenhagen rats were inoculated with $10^5$ Mat Ly Lu-PTHrP cells via i.c. route into the left ventricle. Animals were infused with vehicle alone or the highest dose of PCK3145 (100 μg/kg/day) starting from the day of tumor cell inoculation for 10 days. At the end of these studies, animals were sacrificed, and their vertebrae removed, decalcified, paraffin embedded, and sectioned. Histological sections of vertebrae from animals receiving vehicle alone or the highest dose of PCK3145 were subjected to immunohistochemical analyses using an antibody directed against PTHrP (1-34) as described in “Materials and Methods.” Representative photomicrograph of three such experiments is shown in the top panel at magnification ×100 and in the inset at magnification ×200. Levels of PTHrP production in control (CTL) and experimental metastases were quantified and expressed as total density (bottom panel). Results represent the mean ± SE of five animals in each group in three different experiments. Significant differences in the levels of PTHrP production from control tumor-bearing animals receiving vehicle alone are represented by * ($P < 0.05$).

DISCUSSION

To develop novel therapeutic approaches for late-stage prostate cancer, selection of an appropriate representative in vivo model plays an important role (33). Rat prostate cancer cells Mat Ly Lu-PTHrP therefore allowed us to evaluate the efficacy of PCK3145 on tumor growth and experimental skeletal metastases, which is a hallmark of hormone refractory prostate cancer (9). Additionally, this model is highly beneficial because it permits the determination of biochemical markers of tumor progression like plasma calcium and PTHrP, change in the tumoral PTHrP production, and investigation of the underlying mechanisms responsible for these biological effects. We have previously shown that the naturally occurring protein PSP-94 can reduce prostate cancer growth and hypercalcemia of malignancy in our syngenic in vivo model of rat prostate cancer using Mat Ly Lu-PTHrP cells (24). In the current study, we have used this well-characterized in vivo model of prostate cancer due to its proven efficacy to carry out structure function studies of PSP-94 for identification of the region required for the antitumor effects of this natural peptide. Initially, several peptides corresponding to various amino acid domains of PSP-94 were synthesized and tested. However, both in vitro and in vivo studies clearly demonstrated that the amino acid 31–45 region of PSP-94 (PCK3145) was sufficient to elicit PSP-94-mediated antiproliferative and antitumor effects. The 20-min half-life of PCK3145 (data not shown) is in line with several other peptides that are currently under development for their ability to block tumor growth and was sufficient to cause a significant decrease in tumor growth as reported in this study (34, 35).

Because the major cause of prostate cancer-associated morbidity and mortality is the presence of skeletal metastases, we evaluated the ability of PCK3145 to block the development of experimental skeletal metastases in our in vivo model (7, 8, 24). For these studies, we used the highest dose of PCK3145 (100 μg/kg) that had previously shown maximum efficacy and no noticeable side effects. In this model, use of hind-limb paralysis as a therapeutic end point was found to be insufficient to effectively determine the full effectiveness of PCK3145 on reducing tumor burden in the skeleton. When skeletal tumor burden in experimental animals receiving PCK3145 was determined before any evidence of hind-limb paralysis, a more robust antitumor effect of PCK3145 was observed. These more pronounced antitumor effects at this time point compared with the delay in the development of hind-limb paralysis can be due to rapid tumor growth beyond day 10 of tumor cell inoculation.

The less robust response in blocking skeletal metastases as opposed to decreasing primary tumor volume, when tumor cells were inoculated via s.c. route, can be attributed to decreased bioavailability of PCK3145 to skeleton. Indeed, this is commonly observed when developing any therapeutic strategy for blocking skeletal metastases (36). The major cause of prostate cancer morbidity is bone pain as a result of nerve impingement by skeletal metastatic tumors. An essential component in cancer therapeutics is palliative care and improved quality of life in patients with late-stage cancer. As such, the ability to eliminate or reduce bone pain would be of imperial value. The finding that PCK3145 cannot only reduce the volume of skeletal metastases but also reduce the degree of vertebral column perturbation and spinal cord impingement is highly suggestive of achieving these beneficial effects with this novel peptide in patients with late-stage hormone refractory prostate cancer. Based on these unique characteristics of
small peptides, several small peptides derived from naturally occurring proteins are now being evaluated for their efficacy as potential therapeutic agents for cancer (37, 38). Use of these peptides is particularly attractive because they can be easily synthesized at low cost, they exhibit higher tissue permeability, and they can be conjugated to chemotherapeutic agents for their targeted delivery to avert cytotoxic agents associated side effects (34, 35, 37, 39, 40).

In the current study, we have not only identified the sequence of PSP-94 responsible for its antitumor effects but also showed the ability of PCK3145 to reduce plasma calcium and PTHrP levels. Of

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**Fig. 6.** Effect of PCK3145 on plasma PTHrP and calcium in tumor-bearing animals. A, male Copenhagen rats were inoculated s.c. with $5 \times 10^5$ Mat Ly Lu-PTHrP cells. Starting at the time of tumor cell inoculation, different doses of PCK3145 were administered to the animals for 15 consecutive days as described in “Materials and Methods.” All animals were sacrificed at the end of the study (day 16), and plasma was collected from control (CTL), vehicle-treated animals, and PCK3145-treated animals and analyzed for immunoreactive plasma PTHrP (iPTHrP; A) levels or plasma calcium (B) as described in “Materials and Methods.” Plasma PTHrP and calcium levels in normal non-tumor-bearing animals are also shown (N). Results represent the mean ± SE of five animals in each group in three different experiments. Significant differences from control tumor-bearing animals receiving vehicle alone are represented by * ($P < 0.05$).

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**Fig. 7.** Effect of PCK3145 on PTHrP production by Mat Ly Lu-PTHrP tumors. Male Copenhagen rats were inoculated s.c. with $1 \times 10^6$ Mat Ly Lu-PTHrP cells. Starting at the time of tumor cell inoculation, animals were infused with different doses of PCK3145 for 15 consecutive days as described in “Materials and Methods.” All animals were sacrificed at the end of the study, and primary tumors from control vehicle-treated animals and animals treated with the highest dose of PCK3145 (100 μg/kg/day) were removed, paraffin embedded, and sectioned. Histological sections of tumors from animals receiving vehicle alone (CTL) or the highest dose of PCK3145 were stained with an antibody directed against PTHrP (1–34) as described in “Materials and Methods.” Also shown is a negative control (NC) in which experimental tumor sections were incubated with preabsorbed anti-PTHrP antibody as described in “Materials and Methods” to account for background staining. A representative photomicrograph of three such experiments is shown at magnification $\times 100$ and in the inset as magnification $\times 200$. Results are representative of three animals in each group and three tumor sections that were analyzed from each animal by evaluating at least 10 random fields of observation.
particular note was our finding that although no incremental antitumor effects were observed when experimental animals received 10 or 100 μg/kg PCK3145, the decrease in the level of plasma calcium and PTHrP continued to be of a dose-dependent nature. This change in PTHrP levels was also seen in PTHrP produced by tumor cells in the primary tumors and in experimental skeletal metastases.

Although the exact molecular mechanism responsible for these antitumor effects of PCK3145 remains to be fully elucidated, the current study has provided us with some intriguing clues. These include our studies demonstrating the ability of PCK3145 to induce tumor cell apoptosis. For these studies, we examined both control and experimental primary tumors by TUNEL assay, which exhibited higher levels of DNA fragmentation, a hallmark of apoptosis in tumors from animals receiving PCK3145. These findings were further confirmed by carrying out counterstaining of these histological specimens with Hoechst reagent, which showed increased levels of condensed, apoptotic chromatin in experimental tumors compared with the normal DNA staining pattern observed in tumors of control animals receiving vehicle alone. Although these studies clearly show apoptosis to be one of the mechanisms responsible for the effects of PCK3145, results from this study provide evidence for additional mechanisms that could also play an important role. Previous studies have shown that PSP-94 can down-regulate the levels of follicle-stimulating hormone, which is known to decrease intracellular calcium (41–43). This pathway therefore represents one potential mechanism responsible for the ability of PCK3145 to decrease plasma PTHrP as reported in this study. Additionally, PCK3145 clearly showed its capacity to decrease both tumoral and plasma PTHrP levels. These results suggest that PCK3145 may directly affect the regulation of PTHrP production by tumor cells.

Collectively, results of these studies have demonstrated that amino acids 31–45 of PSP-94 are sufficient for its antitumor effects without any noticeable side effects. This peptide was not only able to reduce tumor growth and experimental skeletal metastasis but also lowered biochemical parameters of late-stage prostate cancer, which provides us with the opportunity to monitor the efficacy of this therapeutic agent at various time points. Although PCK3145 was found to be highly effective, biochemical modification of this peptide including glycosylation, conjugation with polyethylene glycol, or albumin will limit its proteolysis, will increase half-life, and can potentially enhance its biological effects (44–46). Additionally, combination of PCK3145 with chemotherapeutic agents at different doses and time points can provide either additive or synergistic antitumor effects that will add to our existing therapeutic modalities available for men with hormone refractory late-stage prostate cancer to reduce morbidity and mortality.

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A Synthetic 15-mer Peptide (PCK3145) Derived from Prostate Secretory Protein Can Reduce Tumor Growth, Experimental Skeletal Metastases, and Malignancy-Associated Hypercalcemia

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