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Chemopreventive Effects of Pterostilbene in Metastatic Prostate Cancer Cells

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Philadelphia College of Osteopathic Medicine

Biomedical Sciences

**CHEMOPREVENTIVE EFFECTS OF PTEROSTILBENE
IN METASTATIC PROSTATE CANCER CELLS**

A thesis in Biomedical Research by Phillip A. Zook

Submitted in Partial Fulfillment of the Requirements for the Degree of
MS in Biomedical Sciences with concentration in Biomedical Research
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We have read and examined this manuscript and certify that it is adequate in scope and quality as a thesis for this MS degree.

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Listing of Abbreviations

ADT	androgen deprivation therapy
AR	androgen receptor
ARG	androgen-responsive gene
Bcl-2	B-cell lymphoma-2
CDKN1A	cyclin-dependent kinase inhibitor-1A
CDKN1B	cyclin-dependent kinase inhibitor-1B
CRPC	castration-resistant prostate cancer
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
LHRH	luteinizing-hormone-releasing hormone
lncRNA	long non-coding RNA
MMP-9	matrix metalloproteinase-9
PCa	prostate cancer
PCGEM1	prostate cancer gene expression marker-1
PKB	protein kinase B
PRNCR1	prostate cancer non-coding RNA-1
PSA	prostate-specific antigen
PTS	pterostilbene
RSV	resveratrol
SIRT1	silent mating type information regulation 2 homolog-1

Abstract

Recent studies find that pterostilbene (PTS) exhibits more favorable drug properties and similar chemopreventive effects to its structural analogue resveratrol (RSV). However, few studies describe the activity of PTS in prostate cancer (PCa). Here, we conducted cell count experiments to assess the effects of PTS on metastatic PCa cell viability and to compare the potency of PTS to RSV in this respect. We also performed experiments to assess the effects of PTS on the androgen receptor (AR) and AR-mediated events. We used qPCR to measure the mRNA levels of the androgen-responsive gene (ARG), prostate-specific antigen (PSA), and Western blots to assess the expression and subcellular localization of the AR protein in LNCaP cells. We found that PTS inhibited cell viability more potently than RSV in androgen-dependent LNCaP and androgen-independent PC-3 cells. These inhibitory effects were time and dose-dependent and suggest that PTS may provide chemopreventive effects at multiple stages of disease. PTS also inhibited androgen-induced PSA mRNA levels in LNCaP cells. However, this inhibitory effect could not be fully attributed to changes in the androgen-induced expression or nuclear translocation of AR protein. Therefore, further investigation is required to elucidate PTS's effects on AR-mediated events and to assess the clinical applicability of PTS in PCa.

Introduction

Prostate cancer (PCa) is the second-leading cause of cancer-related death among American men and the majority of PCa deaths result from advanced disease [1]. Advanced disease refers to PCa that has metastasized beyond the prostate and its adjacent tissues, including the seminal vesicles, bladder neck, and pelvic lymph nodes [2]. At present, there is no cure for advanced PCa and clinical management of the disease remains problematic [2-4]. Tumors in advanced disease can quickly develop resistance to multiple treatments, and as a result, men who begin advanced PCa therapy experience a median progression-free survival of just 12-18 months [3]. The median overall survival from the start of treatment is less than three years [5]. Therefore, advanced PCa represents a significant concern to men's health in the United States due to the high incidence of disease and its recalcitrance to current therapeutic modalities.

Endocrine-based therapies provide greater clinical benefit to men with advanced PCa than traditional chemotherapeutics, and first-line treatments are agents that impair androgen receptor (AR) activity [4-6]. The AR is a transcription factor that forms complexes with co-regulatory and mediator proteins to regulate the transcription of androgen-responsive genes (ARGs) [2,3]. This activity is required for the normal development and function of the prostate gland [2]. However, dysregulation of AR signaling can lead to abnormal gene expression and contribute to the initiation and progression of PCa [2]. Recent studies indicate that this aberrant signaling plays a significant role in all stages of disease, including castration-resistant PCa (CRPC) [2,3]. Thus, inhibition of AR activity has become the therapeutic mainstay in the treatment of

advanced disease [5].

Androgen deprivation therapy (ADT) employs LHRH (luteinizing-hormone-releasing hormone) antagonists to block the production of testicular androgens and second-line anti-androgens to competitively inhibit the AR [4-6]. This approach provides a clinical benefit to ~80% of men with advanced disease, stalling cancer progression, decreasing the size of the prostate and metastatic tumors, and alleviating symptoms such as bone pain [4-6]. However, ADT is not a curative intervention and disease regression is generally short-lived [4-6]. Subcellular changes to the structure and microenvironment of the AR mediate resistance to ADT agents and as a result, all men with advanced PCa ultimately progress despite treatment [2,3,7,8]. This establishes a clear need for new therapeutic agents in the field of advanced PCa.

Resveratrol (RSV) is a natural polyphenol that displays promising chemopreventive activity in preclinical studies of advanced PCa [9-12]. Experiments performed *in vitro* find that RSV inhibits cell growth and induces apoptosis in a number of metastatic PCa cell lines, including the androgen-dependent LNCaP and androgen-independent PC-3 cell lines [13,14]. This chemopreventive activity has been attributed, at least in part, to RSV's ability to inhibit the androgen-induced expression and function of the AR [15-18]. However, unlike conventional ADT agents, RSV does not inhibit AR activity through ligand deprivation, competitive antagonism, or disruption of AR nuclear translocation [15-18]. Instead, RSV appears to act through a unique mechanism of action that has yet to be fully described. This may be significant as agents demonstrating novel methods of AR inhibition have recently improved the treatment of metastatic disease and emphasized new therapeutic targets in the AR signaling pathway [19-21].

Despite these preclinical results, RSV is not a viable candidate for clinical use in PCa at this time [22]. Pharmacokinetic studies indicate that RSV has a short half-life and limited bioavailability in human subjects [23-26]. RSV is particularly prone to phase II metabolism and is rapidly conjugated to glucuronide and sulfate groups by cells of the liver and small intestine [23]. Thus, recent efforts were made to identify compounds that exhibit similar pharmacologic activity to RSV with more favorable drug properties [27-29]. Pterostilbene (PTS), a natural analogue of RSV, may fulfill these criteria and therefore hold greater potential to elicit chemopreventive effects in human subjects [27,28].

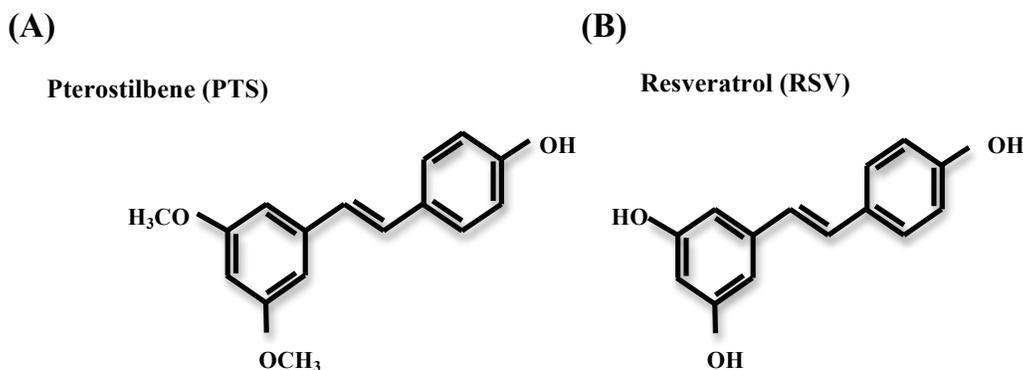


Figure 1. Chemical structures of PTS and RSV.

The chemical structures of PTS and RSV differ by just two methoxy groups (Fig. 1). However, this subtle variation affords PTS a number of pharmacokinetic advantages [29]. PTS exhibits greater lipophilicity, a longer half-life (3-7x), and greater bioavailability (3-4x) than RSV in animal models [22,29,30]. This corresponds to other findings that methylated polyphenols are more efficiently absorbed by the intestines and less vulnerable to hepatic conjugation than their unmethylated counterparts [29]. In the first clinical trial involving PTS, the drug was well tolerated at the highest

administered dose of 250mg/day [31]. Thus, all available evidence suggests that PTS possesses a superior pharmacokinetic profile to its structural analogue, RSV.

Recently, PTS was found to elicit similar, and at times more potent, anti-carcinogenic effects to RSV in preclinical models of colon cancer, hepatocellular carcinoma, and malignant melanoma [9,28]. However, few studies have investigated the chemopreventive effects of PTS in PCa. Chakraborty et al. found that PTS inhibited cell proliferation and induced apoptosis in androgen-independent PC-3 cells [32]. The authors also reported that PTS inhibited molecular targets associated with cell proliferation and survival (PKB, Bcl-2, and MMP-9) and induced factors associated with pro-apoptotic events (Bax and caspases) in these cells [32]. In another study, Wang et al. found that PTS triggered cell cycle arrest in androgen-dependent LNCaP cells, upregulating cyclin-dependent kinase inhibitors, CDKN1A and CDKN1B [33]. However, there is still much to learn about the chemopreventive effects of PTS in PCa and how these effects compare to those demonstrated by RSV.

In the present study, we conducted cell count experiments to assess the effects of PTS on metastatic PCa cell viability and to compare the potency of PTS to RSV in this respect. We found that PTS, like RSV, inhibited the viability of androgen-dependent LNCaP and androgen-independent PC-3 cells in a time and dose-dependent manner. PTS was significantly more potent than RSV in producing these effects. We also performed qPCR and Western blot experiments to assess the effects of PTS on the androgen receptor (AR) and AR-mediated events in LNCaP cells. We found that PTS inhibited androgen-induced mRNA levels of the ARG, prostate-specific antigen (PSA). However, this inhibitory effect could not be fully attributed to changes in the androgen-induced

expression or nuclear translocation of AR protein. Therefore, further investigation is required to better elucidate the effects of PTS on AR-mediated events and to assess the clinical applicability of PTS in PCa.

Materials and Methods

Reagents:

Culture media RPMI 1640 (1x) with L-glutamine and Ham's F-12 (1x) with L-glutamine were purchased from Mediatech, Inc. (Manassas, VA). Phenol Red Free RPMI 1640 (1x) with L-glutamine was purchased from Gibco by Life Technologies (Carlsbad, CA). Media were stored at 4°C. Antibiotic-Antimycotic (100x) (Gibco by Life Technologies) was also stored at 4°C. Premium Fetal Bovine Serum (FBS) and Charcoal/dextran treated FBS were purchased from Atlanta Biological (Oakwood, GA) and stored at -20°C. Trypsin (.25%, 2.21mM EDTA) (Mediatech, Inc.) was also stored at -20°C. LNCaP and PC-3 cells were purchased from the American Type Culture Collection (ATCC) (Manassas, VA). Pterostilbene was purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in DMSO (Sigma-Aldrich) to create a 100mM stock solution. Resveratrol (Sigma-Aldrich) was dissolved in ethanol (Biotechnology Grade Anhydrous-Alcohol, IBI Scientific, Peosta, IA) to create a 200mM stock solution. Synthetic androgen R1881 (Sigma-Aldrich) was dissolved in ethanol to create a 10mM stock solution. Solutions were stored in the dark at -30°C. The qPCR primer pairs for PSA (5'- TCCCCCTGCCCATGTCCCAG -3' and 5'- GTGCAGCACCTGCAGAGGGG -3') and GAPDH (5'- GTC AAGGCTGAGAACGGGAA -3' and 5'- AAATGAGCCCCAGCCTTCTC -3') were purchased from Invitrogen (Carlsbad, CA) and diluted with reaction-grade double distilled (RGDD) water to create 100µM stock solutions. Solutions were stored in the dark at -30°C. AR Antibody (N-20): sc-816 was purchased from Santa Cruz Biotechnology (Dallas, TX) and stored at 4°C. Cytoplasmic

Extraction (CE) buffer was prepared with 10mM HEPES, 60mM KCl, 1mM EDTA, 1mM DTT, 1mM PMSF (Sigma-Aldrich), and 0.075% (v/v) NP40 (Novex by Life Technologies). RIPA buffer was prepared with 50mM Tris-Cl, 150mM NaCl, 1mM PMSF, 0.50% DOC, 0.10% SDS, 1mM DTT (Sigma-Aldrich), and 1% (v/v) NP40 (Novex by Life Technologies). Buffers were stored at 4°C.

Cell Cultures:

LNCaP cells were cultured in Media A (RPMI 1640 with L-glutamine, 10% Premium FBS, 1% Antibiotic-Antimycotic). In some experiments, Media A was replaced by Media B (Phenol Red Free RPMI 1640 with L-glutamine, 10% Charcoal/dextran treated FBS, 1% Antibiotic-Antimycotic). PC-3 cells were cultured in Media C (Ham's F-12 with L-glutamine, 10% Premium FBS, 1% Antibiotic-Antimycotic). Cells were kept in an incubator at 37°C in the presence of 5% CO₂.

Cell Count Methods:

LNCaP cells in Media B were distributed evenly into six 24-well plates (Becton Dickinson Labware, Lincoln Park, NJ) by adding 1mL of a single cell suspension to the wells of each plate in a 6 x 3 arrangement. Plates were then stored in an incubator for 12 hours (37°C, 5% CO₂). After 12 hours, three plates were treated with PTS and/or R1881 as follows: Row 1: "Control" (0µM PTS, 0nM R1881), Row 2: "Androgen Control" (0µM PTS, 10nM R1881), Row 3: 5µM PTS, 10nM R1881, Row 4: 10µM PTS, 10nM R1881, Row 5: 15µM PTS, 10nM R1881, Row 6: 20µM PTS, 10nM R1881. Remaining plates were treated with RSV and/or R1881 as follows: Row 1: "Control" (0µM RSV,

0nM R1881), Row 2: “Androgen Control” (0 μ M RSV, 10nM R1881), Row 3: 10 μ M RSV, 10nM R1881, Row 4: 20 μ M RSV, 10nM R1881, Row 5: 50 μ M RSV, 10nM R1881, Row 6: 100 μ M RSV, 10nM R1881. Following treatment, plates were returned to the incubator.

After 24 hours, one PTS-treated plate and one RSV-treated plate were removed from the incubator for cell counting. Media and detached cells were aspirated from each well and replaced by 100 μ L of trypsin (20°C). After several minutes, the remaining cells were suspended in 500 μ L of Media A (20°C) and distributed into 1.5mL eppendorf tubes (on ice). The Countess Automated Cell Counter (Invitrogen) was used to determine the number of viable cells in a 5 μ L sample drawn from each tube. This procedure was then used to evaluate cell viability after 48 and 72-hour treatments using the remaining two pairs of plates.

Similar methods were used for cell count experiments with PC-3 cells. PC-3 cells were cultured in Media C and distributed into six 24-well plates by adding 1mL of a cell suspension to the wells of each plate in a 5 x 3 arrangement. Plates were then stored in an incubator for 12 hours. After 12 hours, three plates were treated with PTS as follows: Row 1: “Control” (0 μ M PTS), Row 2: 5 μ M PTS, Row 3: 10 μ M PTS, Row 4: 15 μ M PTS, Row 5: 20 μ M PTS. Remaining plates were treated with RSV as follows: Row 1: “Control” (0 μ M RSV), Row 2: 10 μ M RSV, Row 3: 20 μ M RSV, Row 4: 50 μ M RSV, Row 5: 100 μ M RSV. Following treatment, cells were collected and counted as described previously except that Media C was used in place of Media A.

qPCR Methods:

LNCaP cells were suspended in Media A and distributed evenly into six 10mL plates (Chemglass Life Sciences, Vineland, NJ). Plates were stored in an incubator for 24 hours (37°C, 5% CO₂). After 24 hours, Media A was replaced by Media B and plates were returned to the incubator for 6 hours. After 6 hours, plates were treated as follows: Plate 1: “Control” (0µM PTS, 0nM R1881), Plate 2: “Androgen Control” (0µM PTS, 10nM R1881), Plate 3: 5µM PTS, 10nM R1881, Plate 4: 10µM PTS, 10nM R1881, Plate 5: 15µM PTS, 10nM R1881, Plate 6: 20µM PTS, 10nM R1881. After 24 hours, total RNA was isolated from each plate using the RNeasy Plus Micro Kit (Qiagen, Venlo, Limburg, Netherlands), according to the manufacturer’s instructions. RNA was quantified using the NanoDrop 2000c UV-Vis Spectrophotometer and then reverse transcribed using Random 9-mer Primers (Stratagene, La Jolla, CA) and the SuperScript II Reverse Transcriptase Kit (Invitrogen), according to the manufacturer’s instructions. Resulting cDNA was diluted (5x) in RGDD and stored overnight at -30°C. cDNA samples were prepared for qPCR using the reagents and protocol of the SYBR Advantage qPCR Premix (Clontech Laboratories, Inc., Mountain View, CA), and primers pairs for PSA and GAPDH (Invitrogen). Samples were distributed in triplicate into a 96-well qPCR plate (Applied biosystems, Life technologies) and loaded into the ABI PRISM 7000 Sequence Detection System. Using the ABI Software, PSA was labeled as a target gene and GAPDH as an endogenous control. The threshold was set automatically and cycling conditions were entered as follows: Initial Denaturation (1 Rep., 95°C, :30), Two-Step PCR (40 Rep., Denaturation: 95°C, :05; Annealing/Extension: 60°C, :37). The ABI software used the fluorescence data to calculate one relative quantification (RQ)

value per triplicate. RQ values ($2^{-\Delta\Delta C_t}$) from three separate experiments were then used for statistical analysis.

Western Blot Methods:

LNCaP cells were suspended in Media A and distributed evenly into eighteen 10mL plates (one plate to source each nuclear, cytoplasmic, and whole cell lysate for six treatment groups). Plates were stored in an incubator for 24 hours (37°C, 5% CO₂). After 24 hours, Media A was replaced by Media B and plates were returned to the incubator for 6 hours. After 6 hours, plates were treated with different concentrations of PTS and/or 10nM R1881 for 24 hours. After 24 hours, one plate from each treatment group was removed from the incubator. Cells were collected by scraping and transferred to 1.5mL eppendorf tubes. Cell pellets were isolated and whole cell lysates were obtained using RIPA buffer. Cells from the remaining two plates in each treatment group were combined into 1.5mL eppendorf tubes. Cell pellets were isolated and CE buffer was used to separate the contents of each tube into a cytoplasmic extract (supernatant) and nuclear pellet. Cytoplasmic extracts were collected in new 1.5mL eppendorf tubes and placed on ice. Nuclear extracts were obtained from nuclear pellets using RIPA buffer and sonication.

Total protein concentrations from whole cell, nuclear, and cytoplasmic extracts were estimated with the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA). 20µg of total protein from whole cell and cytoplasmic extracts and 60µg of total protein from nuclear extracts were separated on polyacrylamide gels (4% stacking, 10% separating). Proteins were transferred to iBlot Nitrocellulose Transfer

Stacks (Novex by Life Technologies) using the iBlot system (Invitrogen). Ponceau Red stain (Sigma-Aldrich) was used to verify proper transfer of proteins. Blots were blocked in Fast Western Antibody Diluent (Thermo Fisher Scientific) for two hours and incubated overnight with primary AR Antibody (Santa Cruz Biotechnology) at 4°C. Blots were then washed in TBST (Sigma-Aldrich) and incubated with secondary conjugated antibody. For signal development, Clarity Western ECL Substrate (Bio-Rad Laboratories, Hercules, CA) was added to blots according to the manufacturer's instructions. Membranes were then stripped and re-probed for the internal control, β -actin. This experiment was performed three separate times with similar results. Results from the final experiment are displayed in Figure 5.

Statistical Methods:

All statistical analysis was performed using Graphpad Prism 6.0 software (Graphpad Software, Inc., San Diego, CA). Statistical tests applied for each experiment can be found in the corresponding figure legends.

Results

Effects of PTS on Metastatic PCa Cell Viability

We first tested the effects of PTS on metastatic PCa cell viability. Androgen-dependent LNCaP cells were treated with PTS (0-20 μ M) and/or androgen R1881 (10nM) for 24, 48, or 72 hours. Following treatment, the numbers of viable cells in each treatment group were counted as described in the Materials and Methods. Figure 2A shows the time and dose-dependent effects of PTS on LNCaP cell viability. Significant inhibitory effects ($p \leq 0.001$) were observed for PTS at 10 μ M after 24 hours and 5 μ M after 48 and 72 hours.

A similar experiment was performed using androgen-independent PC-3 cells. These cells were treated with different concentrations of PTS (0-20 μ M) for 24, 48, or 72 hours. Following treatment, the numbers of viable cells in each treatment group were counted as described in the Materials and Methods. Figure 2B shows the time and dose-dependent effects of PTS on PC-3 cell viability. In these cells, significant inhibitory effects ($p \leq 0.001$) were observed for PTS at 5 μ M after 48 and 72 hours. No significant effects were observed for PTS on PC-3 cell viability after 24 hours.

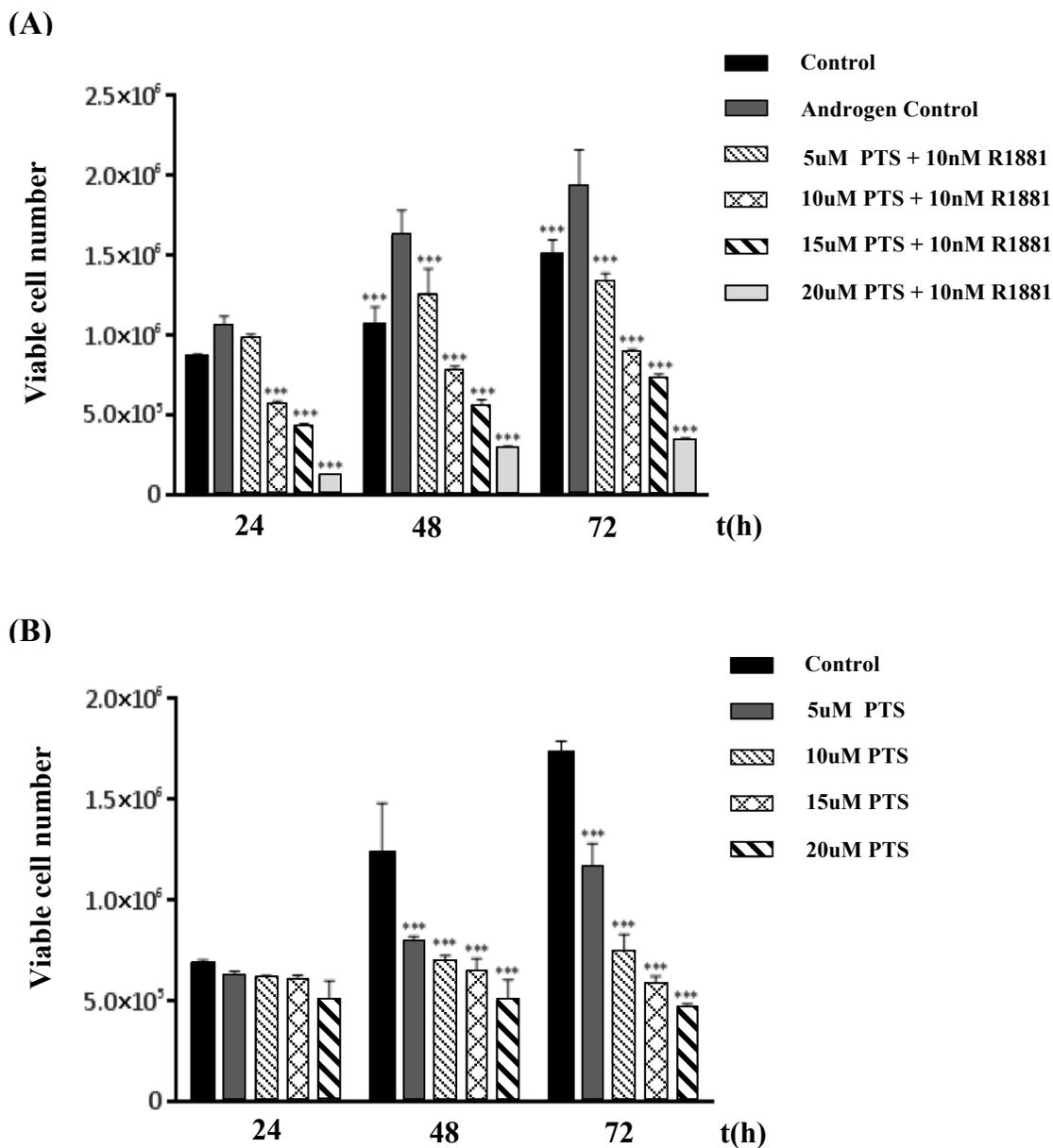


Figure 2. Time and dose-dependent effects of PTS on LNCaP and PC-3 cell viability. LNCaP cells (A) in Media B were treated with different concentrations of PTS and/or 10nM R1881. PC-3 cells (B) in Media C were treated with different concentrations of PTS alone. Viable cell numbers were counted after 24, 48, and 72-hour treatments and the data were expressed as means \pm SEM of three biologic replicates. Two-way ANOVA followed by Tukey test was applied, with intra-group comparisons made to “Androgen Control” (2A) or “Control” (2B). * indicates $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

Cell count experiments were performed with PTS and RSV, as described in the Materials and Methods. In Figure 3, dose-response curves and IC_{50} values were used to illustrate and compare the inhibitory effects of the analogues on LNCaP (Fig. 3A) and PC-3 (Fig. 3B) cell viability after 72 hours. This time point was chosen for display because PTS and RSV demonstrated the most potent effects on cell viability (in both cell lines) after 72 hours. In Figure 3A, the IC_{50} value of PTS was $<10\mu\text{M}$ and was significantly lower ($p<0.001$) than the IC_{50} value of RSV. In Figure 3B, the IC_{50} value of PTS was $<9\mu\text{M}$ and was significantly lower ($p<0.001$) than the IC_{50} value of RSV. This indicates that PTS had more potent inhibitory effects on LNCaP and PC-3 cell viability as compared to RSV.

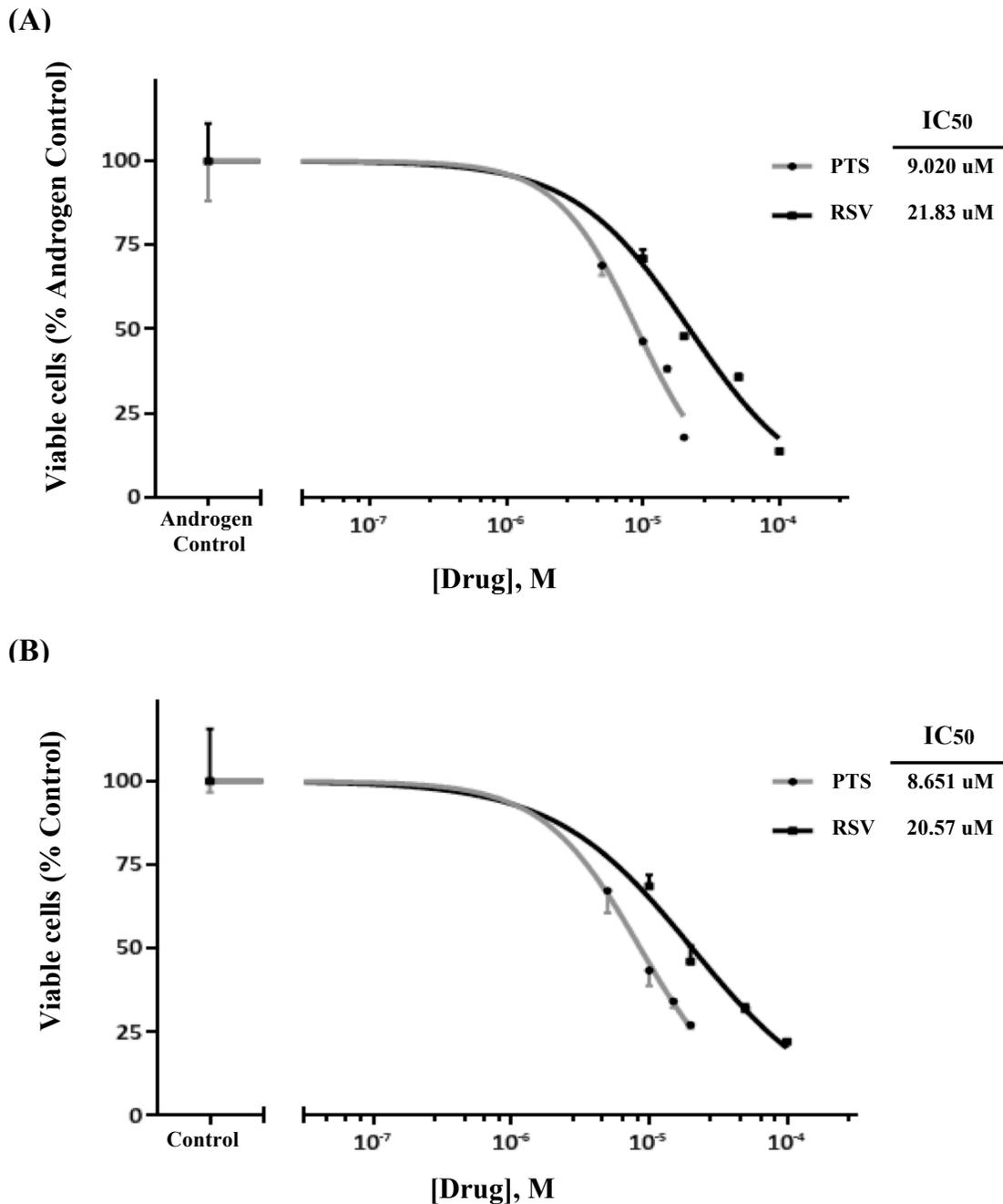


Figure 3. Comparison of PTS and RSV effects on LNCaP and PC-3 cell viability. LNCaP cells (A) in Media B were treated with 10nM R1881 and different concentrations of PTS or RSV. PC-3 cells (B) in Media C were treated with different concentrations of PTS or RSV alone. Viable cell numbers were counted after 72-hour treatments and entered into Graphpad software as means \pm SEM of three biologic replicates. Treatment concentrations were log transformed and viable cell numbers were normalized to represent percentage of “Androgen Control” (3A) or “Control” (3B). Dose-response curves were plotted using a nonlinear regression formula (log (inhibitor) vs. normalized response -- variable slope) and IC₅₀ values were generated from this analysis. IC₅₀ values for PTS and RSV were significantly different ($p < 0.001$).

Effects of PTS on PSA mRNA Levels in Metastatic PCa Cells

To clarify whether the chemopreventive effects of PTS in metastatic PCa cells could be mediated, at least in part, through effects on AR-mediated events, LNCaP cells were treated with PTS (0-20 μ M) and/or androgen R1881 (10nM) for 24 hours. After 24 hours, cells were collected and prepared for SYBR qPCR as described in the Materials and Methods. In Figure 4, the effects of PTS on PSA mRNA levels in LNCaP cells are shown. Treatment with PTS resulted in a dose-dependent inhibition of androgen-induced PSA mRNA levels in these cells. Significant inhibitory effects ($p \leq 0.01$) were observed for PTS at 5 μ M after 24 hours and these results are consistent with an inhibition of AR-mediated events.

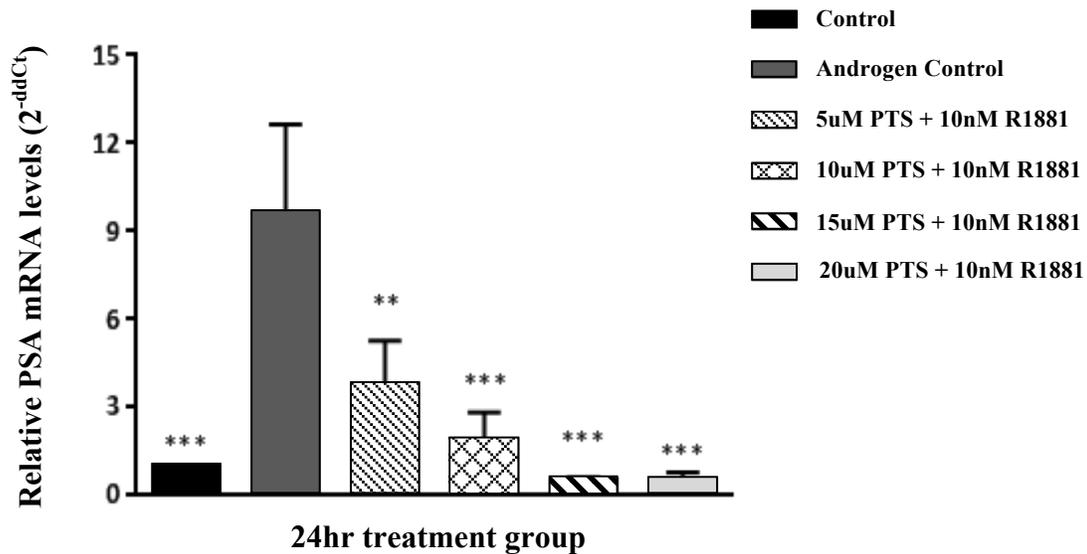
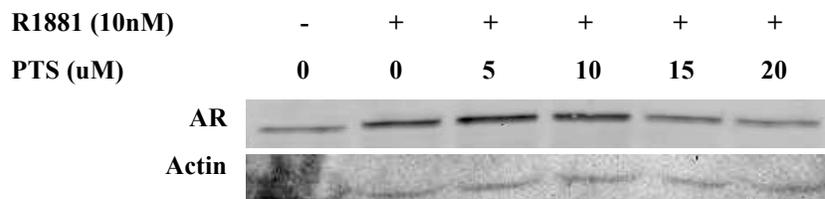


Figure 4. Effects of PTS on androgen-induced PSA mRNA levels in LNCaP cells. LNCaP cells in Media B were treated with different concentrations of PTS and/or 10nM R1881 for 24 hours. Cells were harvested and total RNA was purified and reverse transcribed. Relative quantification (RQ) values for PSA mRNA were determined using qPCR as described in the Materials and Methods. Relative PSA mRNA levels were expressed as means \pm SEM of three separate experiments. One-way ANOVA followed by Tukey test was applied, with statistical comparisons made to “Androgen Control.” * indicates $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

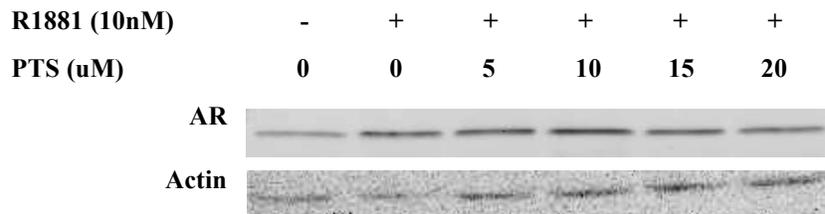
Effects of PTS on the AR Protein in Metastatic PCa Cells

To determine if PTS's inhibition of androgen-induced PSA mRNA levels could be attributed to changes in the expression or nuclear translocation of AR protein, LNCaP cells were treated with different concentrations of PTS (0-20 μ M) and/or 10nM R1881 for 24 hours. After 24 hours, cells were harvested and a whole cell lysate (Fig. 5A), cytoplasmic extract (Fig. 5B), and nuclear extract (Fig. 5C) were obtained. Proteins were separated on polyacrylamide gels and Western blots were performed as described in the Materials and Methods. This experiment was repeated three times with similar results. In Figure 5A, we observed that treatment with PTS had no effect on total androgen-induced AR protein expression at concentrations below 10 μ M. In Figures 5B and 5C, we observed that treatment of LNCaP cells with PTS did not effect androgen-induced AR nuclear translocation. These results suggest that PTS's inhibition of androgen-induced PSA mRNA levels cannot be fully attributed to effects on AR protein expression or to any effects on AR nuclear translocation.

(A)



(B)



(C)

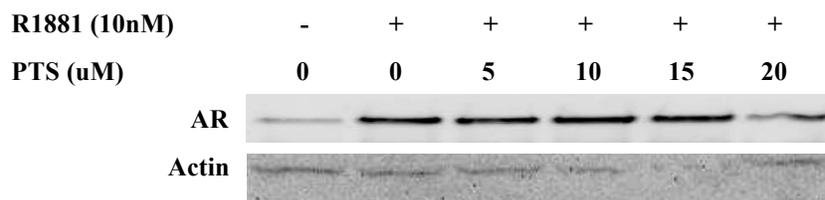


Figure 5. Effects of PTS on androgen-induced expression and localization of AR protein in LNCaP cells. LNCaP cells in Media B were treated with different concentrations of PTS and/or 10nM R1881 for 24 hours. Whole cell (A), cytoplasmic (B), and nuclear (C) extracts were obtained and separated on polyacrylamide gels (4% stacking, 10% separating). Western blots were performed with anti-AR antibody before membranes were stripped and re-probed for β -actin (internal control). Experiment was performed three separate times with similar results.

Discussion

RSV demonstrates unique chemopreventive effects in pre-clinical studies of PCa, but low bioavailability renders the compound ineffectual in human subjects [23-25]. Recently, a structural analogue called PTS was found to possess a superior pharmacokinetic profile to RSV while maintaining similar pharmacologic activity [9,22,28-30]. However, the chemopreventive activity of PTS in PCa has not been well established. In the current study, we assessed the chemopreventive activity of PTS in PCa by investigating its effects on cell viability and AR-mediated events in metastatic PCa cells.

We found that PTS inhibited the viability of both androgen-dependent and androgen-independent PCa cells. These findings are consistent with the results reported by Wang and Chakraborty et. al, and serve to validate PTS's chemopreventive activity in metastatic PCa [32,33]. However, the chemopreventive effects of PTS may not be limited to advanced disease. LNCaP cells, while of metastatic lineage, are commonly used in research to represent earlier stages of disease. This is due to the androgen-dependent status of these cells, as well as their relatively low tumorigenicity in comparison to other metastatic PCa cell lines [34]. Thus, while only metastatic PCa cells were used in this study, it is important to note that PTS may provide chemopreventive effects at multiple stages of disease.

In addition to validating the general chemopreventive activity of PTS in PCa, our cell count experiments also allowed us to compare the effects of PTS to its well-studied structural analogue, RSV. We found that both analogues had time and dose-dependent

effects on LNCaP and PC-3 cell viability. Benitez et. al originally reported that RSV produced time and dose-dependent effects on PCa cell viability, but this is the first study to demonstrate that PTS works in similar fashion [14]. Both analogues demonstrated the most potent inhibitory effects after 72 hours and their respective dose-response curves and IC₅₀ values were compared at this time point in Figure 3. We found that PTS inhibited cell viability with greater potency than RSV in both cell lines, demonstrating significant inhibitory effects at low micromolar concentrations. Dose-escalation studies involving PTS have yet to be performed in human subjects, but concentrations of unchanged RSV have been measured in human serum at ~5 μ M [25,26]. Extrapolating from PTS's superior bioavailability to RSV in animal models, it is therefore possible that the concentrations of PTS used in this study may be attainable in human serum [25,29,30]. As a result, PTS may hold greater potential to elicit chemopreventive effects in humans than RSV.

To further clarify *how* PTS produces chemopreventive effects in metastatic PCa cells, we investigated its effects on the AR and AR-mediated events. The AR plays a significant role in the initiation and progression of PCa and this pathway remains the primary therapeutic target in the treatment of advanced disease [5]. We also chose to investigate the effects of PTS on this signaling pathway because the chemopreventive effects of RSV are known to be mediated, at least in part, by an inhibition of AR expression and function [15-18]. To assess the effects of PTS on the AR and AR-mediated events we performed experiments in androgen-dependent LNCaP cells. Androgen-dependent cells rely on exogenous androgens for survival and are highly sensitive to changes in exogenous androgen levels. As a result, any effects of PTS on the

AR and AR-mediated events can be easily discerned in LNCaP cells.

Examples of androgen-induced effects on LNCaP cells can be found in the results of each experiment. In Figure 2A, “Androgen Control” groups (0 μ M PTS, 10nM R1881) demonstrated significantly ($p \leq 0.001$) greater numbers of viable cells than “Control” groups (0 μ M PTS, 0nM R1881) at the 48 and 72 hour time points. “Androgen Control” groups also showed significantly greater ($p \leq 0.05$) PSA mRNA levels (Fig. 4) and whole cell AR protein expression (Fig. 5) as compared to their corresponding “Control” groups after 24 hours. These results are to be expected as AR expression and function are enhanced following exposure of LNCaP cells to exogenous androgen supplies [15,16]. Androgen treatment results in greater cell viability (Fig. 2A) because AR activity plays a significant role in the survival and proliferation of PCa cells [33].

To determine if PTS affects androgen-induced PSA mRNA levels in LNCaP cells we performed qPCR experiments. As shown in Figure 4, PTS inhibited androgen-induced PSA mRNA levels in a dose-dependent manner and significant inhibitory effects were observed for PTS at 5 μ M after 24 hours. These effects on the mRNA levels of PSA, a well-known ARG, are consistent with an inhibition of AR-mediated events. As previously mentioned, the AR plays a significant role in the initiation and progression of PCa and represents the primary therapeutic target in the treatment of advanced disease [2,4-6]. Thus, the chemopreventive effects of PTS in metastatic PCa cells may be due, at least in part, to effects on AR-mediated events.

To gain more specific information regarding the effects of PTS on the AR and AR-mediated events, we performed Western blot experiments in LNCaP cells. In Figure 5, we found that PTS had no effect on androgen-induced AR nuclear translocation. We

also found that total AR protein levels were unaffected by treatment with PTS at concentrations up to 10 μ M (Fig. 5A). Based on these results, we cannot fully attribute the inhibitory effects of PTS on PSA mRNA levels to effects on total AR protein levels or to any effects on AR nuclear translocation. Interestingly, we did observe a decline in androgen-induced AR protein expression when cells were treated with PTS at 15-20 μ M (Fig. 5A). It has previously been reported that RSV triggers degradation of the AR protein at high concentrations (~50 μ M) [15-17]. It is possible that PTS, like RSV, also has post-translational effects on the AR protein at high concentrations. However, this is only a hypothesis and effects on androgen-induced AR protein expression do not appear to play a significant role in PTS's inhibition of PSA mRNA levels. Thus, while PTS appears to inhibit AR-mediated events in metastatic PCa cells, the full mechanism(s) behind this activity have yet to be fully elucidated.

Recent advances in the molecular mechanisms of AR function indicate that two long non-coding RNAs (lncRNAs), PCGEM1 and PRNCR1, serve as integral components of an adaptor complex formed between an AR-bound enhancer site and the promoter region of an ARG [35,36]. Furthermore, interactions between these lncRNAs and the AR are affected by AR acetylation status, which is partially determined by the deacetylase, SIRT1 [36-38]. RSV is a well-established SIRT1 activator [38]. Given the structural similarity of PTS to RSV, as well as the mounting evidence of its similar pharmacologic activity, we speculate that PTS may also serve as a SIRT1 activator. Therefore, we postulate that PTS may inhibit AR transcriptional activity by activating SIRT1, enhancing AR deacetylation, and inhibiting the interaction of lncRNAs and the AR. This could inhibit the formation of adaptor complexes utilized by wild-type and

ligand-independent ARs to facilitate the transcription of ARGs [36]. In summary, PCGEM1 and PRNCR1 may represent new therapeutic targets in advanced PCa and it would be interesting to investigate whether any correlation exists between PTS's inhibitory effects on AR-mediated events and the expression and/or function of these lncRNAs.

We have so far highlighted the observed and potential effects of PTS on the AR and AR-mediated events in androgen-dependent LNCaP cells, but it should also be noted that PTS demonstrated chemopreventive activity in PC-3 cells. PC-3 cells are androgen-independent cells that are insensitive to changes in exogenous androgen supplies and are capable of survival in androgen-depleted environments [39]. While results from recent studies indicate that these cells also express the AR, they do so at much lower levels and demonstrate significantly less AR-mediated activity than LNCaP cells [39]. Thus, AR signaling may play a far less prominent role in cell survival and proliferation in these cells as compared to LNCaP cells. In our cell count experiments, we found that PTS inhibited the viability of LNCaP and PC-3 cells with very similar potency. These results suggest that the chemopreventive effects of PTS are not entirely mediated through effects on the AR. This further supports the hypothesis that PTS acts in similar fashion to RSV, which demonstrates a number of AR-independent effects in PCa [27]. The AR-independent effects of PTS should also be investigated further as they will likely improve our understanding of PTS's pharmacologic activity in PCa, and may also implicate PTS as a potential chemopreventive agent in other cancers.

In conclusion, PTS demonstrates potent chemopreventive effects in both androgen-dependent and androgen-independent metastatic PCa cells. We believe that

these effects are mediated, at least in part, through an inhibition of AR signaling. PTS does not inhibit AR signaling by affecting AR nuclear translocation and the mechanism behind this inhibition has yet to be fully elucidated. Future investigation may reveal that PTS modulates AR activity like its structural analogue RSV. This would mean that the chemopreventive effects of PTS are at least partially mediated through a mechanism of action that is unique to any current ADT agent. A better understanding of this mechanism could provide insight into new therapeutic targets in the AR signaling pathway. In closing, PTS should be investigated further to better elucidate its chemopreventive effects in PCa cells and to assess its potential for clinical applicability in PCa.

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