

Research Paper

Anti-Androgen Receptor Signaling and Prostate Cancer Inhibitory Effects of Sucrose- and Benzophenone-Compounds

Zhe Wang,¹ Hyo-Jeong Lee,^{1,2} Lei Wang,¹ Cheng Jiang,¹ Nam-In Baek,³ Sung-Hoon Kim,^{1,2,4} and Junxuan Lü^{1,4}

Received November 11, 2008; accepted January 13, 2009; published online March 6, 2009

Purpose. Novel agents that target multiple aspects of androgen receptor (AR) signaling are desirable for chemoprevention and treatment of prostate cancer (PCa). We aimed to identify compounds isolated from medicinal herbs as such drug candidates.

Methods. In the LNCaP human androgen sensitive PCa cell model, we tested five compounds purified from *Lindera fruticosa* Hemsley in the range of 10–50 μ M for growth inhibition and AR-prostate specific antigen (PSA) suppressing potency. We determined the relationship between these activities and P53 tumor suppressor protein activation and apoptotic cleavage of PARP. We compared these compounds to the anti-androgen drug Casodex/bicalutamide to identify mechanistic novelty.

Results. Among 3 sucrose compounds, beta-D-(3,4-di-sinapoyl)fructofuranosyl-alpha-D-(6-sinapoyl) glucopyranoside decreased AR and PSA mRNA and protein levels in LNCaP cells and inhibited androgen-stimulated AR translocation from the cytosol to the nucleus. This compound also increased P53 Ser¹⁵ phosphorylation and PARP cleavage in LNCaP cells, but required higher dosage than for suppressing AR-PSA. Interestingly, this compound did not inhibit the growth of RWPE-1 non-transformed prostate epithelial cells. The benzophenone compound 2-methoxy-3,4-(methylenedioxy) benzophenone suppressed PSA and AR in LNCaP cells without apoptosis.

Conclusions. Our data support novel anti-AR actions of these herbal compounds distinct from Casodex and merit further investigation as drug candidates.

KEY WORDS: androgen receptor; benzophenone; prostate cancer; PSA; sucrose derivatives.

INTRODUCTION

Prostate cancer (PCa) is the second leading cause of cancer death in American men and is responsible for an estimated 30,000 deaths per year (1). The androgen receptor (AR) is a ligand-dependent transcription factor, mediating the genomic effects of androgen action in the prostate and androgen-responsive cells (2,3). The importance of androgen and AR in PCa is supported by the observations that PCa rarely occurs in eunuchs or in men with deficiency in 5 α -reductases, the enzymes that convert testosterone to its active metabolite 5 α -dihydrotestosterone (DHT) (4). A clinical trial with finasteride (Proscar), which inhibits 5 α -reductase-2 within the prostate gland, had shown a significant reduction of total PCa incidence (5). However, PCa that developed in subjects in the intervention group appeared to be more

advanced in tumor stages than those from the placebo group, raising doubt about the overall survival benefit of this single-target approach at reducing DHT availability for PCa chemoprevention. In addition, serious side effects of finasteride, including mode swings, hot flushes and impotence, significantly limit its long-term use in young men for the modification of their potential risk of PCa. Novel agents that target multiple aspects of androgen-AR signaling will be desirable for chemoprevention and treatment of PCa as either a single modality or in combination with finasteride and other existing hormone ablation drugs.

Medicinal herbs are a rich source of novel anti-cancer agents. In collaborative work aimed at developing safe and efficacious herbal compounds for PCa chemoprevention, we focused on medicinal herbal phytochemicals that target AR signaling. We have reported the discovery of pyranocoumarin compound decursin and its isomer decursinol angelate from the Korean herb *Angelica Gigas* Nakai as members of a novel class of anti-AR signaling compounds distinct from the clinically used androgen antagonist drug bicalutamide Casodex (6,7).

Lindera fruticosa Hemsley is a shrub that grows in China, Nepal, India and Ethiopia (8,9). Its fruits and roots have been used in folk remedies for gastric diseases and inflammation. The following compounds were recently identified by the Baek group from its root (8,9): sucrose-ester compounds (serial testing number) #14 (UNB-2), alpha-D-(3-sinapoyl)

¹ Hormel Institute, University of Minnesota, 801 16th Avenue, N.E., Austin, Minnesota 55912, USA.

² Cancer Preventive Material Development Research Center and Institute, College of Oriental Medicine, Kyunghee University, 1 Hoegi-dong, Dongdaemun-gu, Seoul, 131-701, Republic of Korea.

³ Graduate School of Biotechnology and Plant Metabolism Research Center, Kyunghee University, Yongin, Republic of Korea.

⁴ To whom correspondence should be addressed. (e-mail: sungkim7@khu.ac.kr; jlu@hi.umn.edu)

fructofuranosyl- α -D-(6-sinapoyl)glucopyranoside; **#17** (UNE-4-8-2), β -D-(3,4-di-sinapoyl)fructofuranosyl- α -D-(6-sinapoyl)glucopyranoside; **#18** (UNE-7-4), β -D-(3-sinapoyl)fructofuranosyl- α -D-(6-sinapoyl)glucopyranoside and two benzophenone compounds 2-methoxy-3,4-(methylenedioxy)benzophenone (**#15**, UNE-2-6) and benzyl-2-hydroxy-6-methoxybenzoate (**#16**, UNE-2-7-3) (for structures, see Fig. 1). These compounds have been previously reported in the African herb *Securidaca longipedunculata* Fresen and Chinses *Securidaca inappendiculata* HASSK (10–12). These herbs are used in folk herbal medicine as anti-inflammatory, antibacterial, and anti-rheumatism ingredients (10–12). The Baek's group has shown an inhibitory activity of **#17** and **#18** on osteoclast differentiation *in vitro* (8). The same group evaluated the benzophenone compounds for the inhibitory effects on human acyl-CoA:

cholesterol acyltransferase activity and on the *in vitro* oxidation of low-density lipoprotein (9). However, none of these compounds has been evaluated for anti-cancer or anti-AR signaling activities.

To identify novel anti-AR signaling herbal compounds, we evaluated these sucrose derivatives (phenolic glycosides) and benzophenone compounds for their suppressing activities in LNCaP human androgen sensitive prostate cancer cells on the expression of the best known AR-target gene product prostate specific antigen (PSA) in comparison with decursin, which we have discovered as a novel anti-AR agent distinct from Casodex (6,7). We report here, for the first time, the identification of **#17**, β -D-(3,4-di-sinapoyl)fructofuranosyl- α -D-(6-sinapoyl)glucopyranoside; and **#15**, 2-methoxy-3,4-(methylenedioxy)benzophenone as novel anti-AR signaling agents.

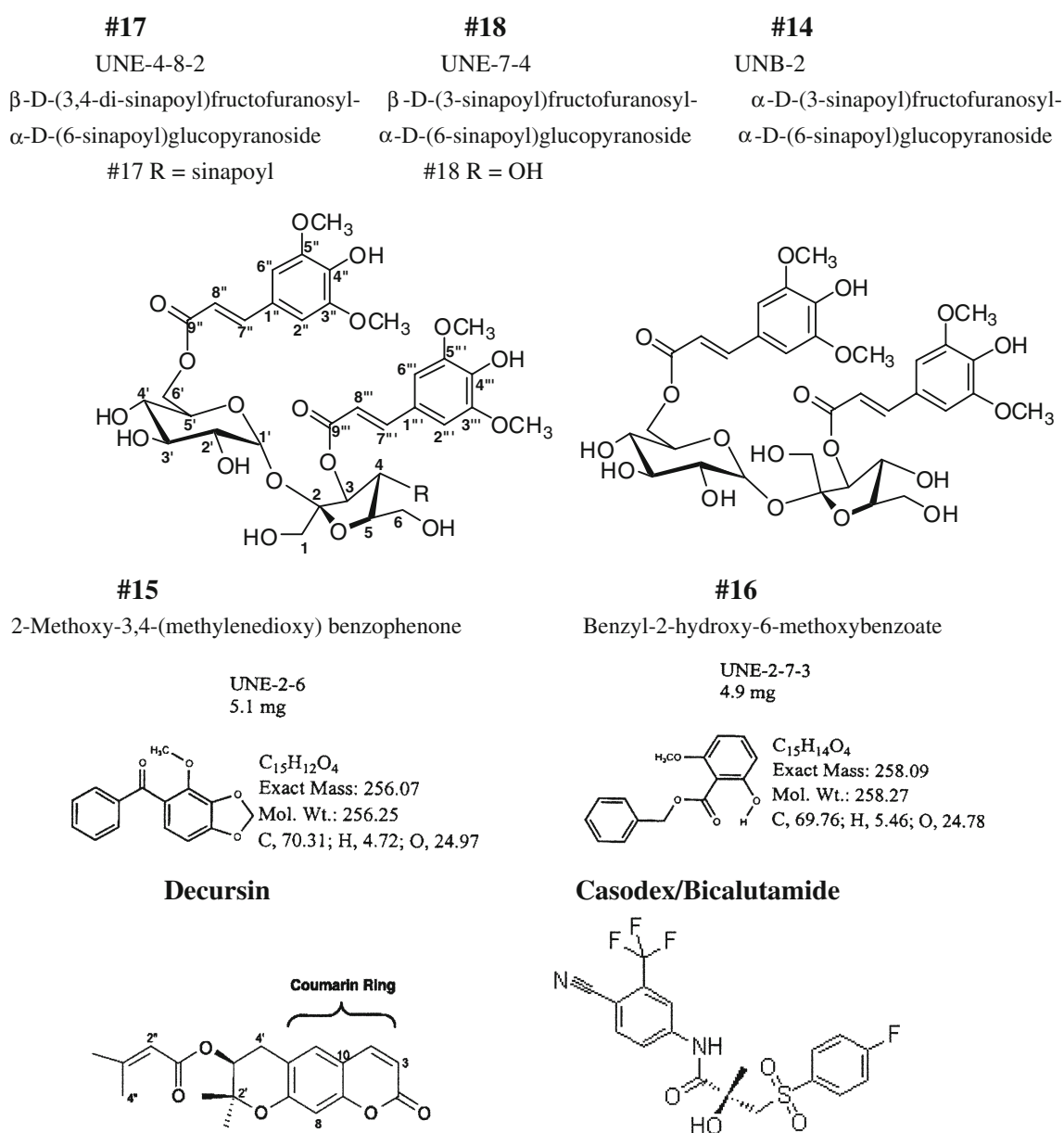


Fig. 1. Structures of test compounds along with those for a novel anti-AR compound decursin and the clinically used androgen antagonist drug Casodex/bicalutamide.

MATERIALS AND METHODS

Extraction and isolation of test compounds. Similar schema as recently reported (8,9) were followed. Dried, powdered roots (1 kg) of *Lindera fruticosa* Hemsley were extracted with 80% aqueous methanol (MeOH-H₂O) (20 l × 3), and concentrated *in vacuo*. The extracts were partitioned with H₂O (2 l), EtOAc (2 l × 3), and n-BuOH (2 l × 3). The concentrated EtOAc fraction (UNE, 14 g) was subjected to silica gel column chromatography (150 g, Φ 6.5 × 12 cm) and eluted with a gradient of CHCl₃-MeOH (10:1 → 7:1, 1 l of each), resulting in 12 fractions (UNE1–UNE12). Fraction UNE4 [1.3 g, Ve/Vt (elution volume/total volume) 0.15–0.18] was subjected to silica gel column chromatography (150 g, Φ 6 × 10 cm) and eluted with CHCl₃-MeOH (10:1 → 8:1 → 6:1 → 4:1, 2.2 → 1.8 → 1.4 → 1.0 l of each), yielding compound **#17** [UNE-4-8-2, 231 mg, Ve/Vt 0.40–0.70; TLC (Keisegel 60 F₂₅₄) R_f 0.4, CHCl₃-MeOH, 5:1]. Fraction UNE7 (584 mg, Ve/Vt 0.27–0.53) was separated by silica gel column chromatography (75 g, Φ 3.5 × 15 cm) and eluted with CHCl₃-MeOH (5:1, 1.8 L), yielding compound **#18** [UNE-7-4, 210 mg, Ve/Vt 0.20–0.30; TLC (Keisegel 60 F₂₅₄) R_f 0.5, CHCl₃-MeOH, 3:1]. The concentrated n-BuOH fraction (UNB, 14 g) was subjected to silica gel column chromatography (150 g, Φ 6 × 14.5 cm) and eluted with a gradient of CHCl₃-MeOH-H₂O (7:3:1 → 6:4:1, 2.2 l of each lower layer), resulting in 10 fractions (UNB1–UNB10) including a purified glycoside, compound **#14** [UNB-2, 6 g, Ve/Vt 0.05–0.15; TLC (Keisegel 60 F₂₅₄) R_f 0.7, CHCl₃-MeOH-H₂O, 6:4:1, lower layer].

β-D-(3,4-disinapoyl)fructofuranosyl-α-D-(6-sinapoyl)-glucopyranoside (#17**):** a yellow amorphous powder: $[\alpha]_D^{25} -45.0^\circ$ (c 0.19, MeOH); IR (CaF₂ window in MeOH) ν_{\max} 3,383, 2,937, 2,841, 1,731, 1,658, 1,470, 1,281, 1,071 cm⁻¹; mp 124–125°C; neg. FABMS m/z 959 [M-H]⁻; ¹H-NMR (400 MHz, CD₃OD, δ) 4.22 (ddd, J=8.0, 7.6, 4.0 Hz, H-5), 3.75 (dd, J=8.8, 8.8 Hz, H-3'), 5.71 (dd, J=8.0, 8.0 Hz, H-4), 4.01 (dd, J=12.0, 7.6 Hz, H-6a), 4.21 (dd, J=10.8, 8.0 Hz, H-6'b), 3.34 (dd, J=8.8, 8.8 Hz, H-4'), 7.58 (d, J=16.0 Hz, H-7'''), 7.54 (d, J=16.0 Hz, H-7''), 7.43 (d, J=16.0 Hz, H-7'''), 4.45 (br dd, J=8.8, 8.0 Hz, H-5'), 3.68 (d, J=13.2 Hz, H-1a), 3.65 (d, J=13.2 Hz, H-1b), 6.47 (d, J=16.0 Hz, H-8'''), 6.44 (d, J=16.0 Hz, H-8''), 6.24 (d, J=16.0 Hz, H-8'''), 3.94 (dd, J=12.0, 4.0 Hz, H-6b), 3.53 (dd, J=8.8, 3.6 Hz, H-2'), 5.87 (d, J=8.0 Hz, H-3), 4.79 (br d, J=10.8 Hz, H-6'a), 5.57 (d, J=3.6 Hz, H-1'), 6.85 (s, H-2''), 6.76 (s, H-2'''), 6.69 (s, H-2''), 6.85 (s, H-6''), 6.76 (s, H-6'''), 6.69 (s, H-6'''), 3.74 (6H, s, H-OCH₃), 3.79 (6H, s, H-OCH₃), 3.80 (6H, s, H-OCH₃), ¹³C-NMR (100 MHz, CD₃OD, δ) 169.0 (C-9'''), 167.8 (C-9''), 167.5 (C-9'), 149.1 (C-3''/5''), 149.0 (C-3'''/5'''), 148.9 (C-3'''/5'''), 148.2 (C-7''), 148.0 (C-7'''), 147.0 (C-7'), 139.4 (C-4''), 139.4 (C-4'''), 139.2 (C-4'''), 126.4 (C-1''), 126.2 (C-1'''), 115.8 (C-8'''), 114.9 (C-8''), 114.5 (C-8'), 106.8 (C-2''/6''), 106.8 (C-2'''/6'''), 106.6 (C-2''/6'''), 105.2 (C-2), 92.9 (C-1'), 82.7 (C-5), 77.0 (C-3), 76.4 (C-4), 74.9 (C-3'), 72.9 (C-2'), 71.9 (C-4'), 72.7 (C-5'), 65.1 (C-1), 65.8 (C-6'), 63.9 (C-6), 126.1 (1'''), 56.7 (×4, C-OCH₃), 56.6 (×2, C-OCH₃).

β-D-(3-sinapoyl)fructofuranosyl-α-D-(6-sinapoyl)glucopyranoside (#18**):** yellow amorphous powder: $[\alpha]_D^{25} -32.0^\circ$ (c 0.27, MeOH); IR (CaF₂ window in MeOH) ν_{\max} 3,384, 2,936, 1,694, 1,629, 1,603, 1,514, 1,456, 1,282, 1,112, 1,048 cm⁻¹; mp 138–141°C; neg. FABMS m/z 753 [M-H]⁻;

¹H-NMR (400 MHz, CD₃OD, δ) 3.74 (dd, J=10.0, 8.0 Hz, H-3'), 4.01 (ddd, J=8.0, 7.6, 4.0 Hz, H-5), 4.21 (dd, J=10.8, 8.0 Hz, H-6'b), 3.89 (dd, J=11.2, 7.6 Hz, H-6b), 7.64 (d, J=16.0 Hz, H-7''), 7.56 (d, J=16.0 Hz, H-7'''), 3.34 (dd, J=8.0, 9.2 Hz, H-4'), 4.53 (dd, J=8.0, 8.0 Hz, H-4), 4.28 (br dd, J=9.2, 8.0 Hz, H-5'), 3.66 (d, J=12.8 Hz, H-1a), 3.62 (d, J=12.8 Hz, H-1b), 3.52 (dd, J=10.0, 3.6 Hz, H-2'), 6.44 (d, J=16.0 Hz, H-8''), 6.43 (d, J=16.0 Hz, H-8'''), 3.94 (dd, J=11.2, 4.0 Hz, H-6a), 5.54 (d, J=8.0 Hz, H-3), 4.79 (br d, J=10.8 Hz, H-6'a), 5.53 (d, J=3.6 Hz, H-1'), 6.85 (s, H-2''), 6.82 (s, H-2'''), 6.85 (s, H-6''), 6.82 (s, H-6'''), 3.83 (6H, s, H-OCH₃), 3.80 (6H, s, H-OCH₃), ¹³C-NMR (100 MHz, CD₃OD, δ) 168.0 (C-9''), 167.2 (C-9'''), 149.1 (C-3''/5''), 148.1 (C-3'''/5'''), 146.9 (C-7''), 146.2 (C-7'''), 138.3 (C-4''), 138.1 (C-4'''), 125.4 (C-1''), 125.3 (C-1'''), 114.6 (C-8''), 114.2 (C-8'''), 105.8 (C-2''/6''), 105.6 (C-2'''/6'''), 105.2 (C-2), 92.4 (C-1'), 82.4 (C-5), 79.2 (C-3), 74.9 (C-3'), 72.9 (C-2'), 74.0 (C-4), 71.7 (C-4'), 72.3 (C-5'), 65.7 (C-1), 65.4 (C-6'), 63.6 (C-6), 56.6 (×2, C-OCH₃), 55.7 (×2, C-OCH₃).

α-D-(3-sinapoyl)fructofuranosyl-α-D-(6-sinapoyl)glucopyranoside (#14**):** yellow amorphous powder: $[\alpha]_D^{26} -30.5^\circ$ (c 0.26, MeOH); IR (CaF₂ window in MeOH) ν_{\max} 3,398, 2,938, 1,699, 1,630, 1,602, 1,514, 1,456, 1,426, 1,376, 1,336, 1,112, 1,052 cm⁻¹; mp 129–130°C; neg. FABMS m/z 753 [M-H]⁻; ¹H-NMR (400 MHz, CD₃OD, δ) 7.66 (d, J=16.0 Hz, H-7''), 6.46 (d, J=16.0 Hz, H-8''), 7.58 (d, J=16.0 Hz, H-7'''), 6.45 (d, J=16.0 Hz, H-8'''), 3.62 (d, J=12.0 Hz, H-1a), 3.57 (d, J=12.0 Hz, H-1b), 4.67 (br d, J=11.6 Hz, H-6'a), 4.28 (br dd, J=8.8, 7.6 Hz, H-5'), 5.51 (d, J=7.6 Hz, H-3), 4.21 (dd, J=11.6, 7.6 Hz, H-6'b), 3.97 (ddd, J=8.0, 7.6, 3.2 Hz, H-5), 5.52 (d, J=3.6 Hz, H-1'), 3.66 (dd, J=9.2, 8.0 Hz, H-3'), 3.89 (dd, J=11.2, 7.6 Hz, H-6b), 3.47 (dd, J=9.2, 3.6 Hz, H-2'), 3.94 (dd, J=11.2, 3.2 Hz, H-6a), 3.32 (dd, J=8.0, 8.8 Hz, H-4'), 4.50 (dd, J=8.0, 8.0 Hz, H-4), 6.92 (s, H-2''), 6.92 (s, H-6''), 6.88 (s, H-2'''), 6.88 (s, H-6'''), 3.87 (6H, s, H-OCH₃), 3.84 (6H, s, H-OCH₃), ¹³C-NMR (100 MHz, CD₃OD, δ) 169.1 (C-9'''), 168.2 (C-9''), 149.3 (C-3''/5''), 149.3 (C-3'''/5'''), 147.9 (C-7''), 147.3 (C-7'''), 139.5 (C-4''), 139.4 (C-4'''), 126.6 (C-1''), 126.5 (C-1'''), 115.8 (C-8''), 115.4 (C-8'''), 107.0 (C-2''/6''), 106.8 (C-2'''/6'''), 104.8 (C-2), 92.6 (C-1'), 84.3 (C-5), 79.1 (C-3), 75.1 (C-3'), 73.1 (C-2'), 74.1 (C-4), 71.9 (C-4'), 72.5 (C-5'), 65.7 (C-1), 65.7 (C-6'), 63.8 (C-6), 55.7 (×4, C-OCH₃).

The isolation and identification of two benzophenone compounds 2-methoxy-3,4-(methylenedioxy)benzophenone (**#15**, **UNE-2-6**, same as compound 1 in ref. (9) and benzyl-2-hydroxy-6-methoxybenzoate (**#16**, **UNE-2-7-3**, same as compound 6 in ref. (9) have been reported by the Baek's group. The structures of these compounds, decursin and Casodex were shown in Fig. 1. These compounds were dissolved in DMSO as stock solutions for treating cultured cells.

Other chemicals and reagents. The following materials were purchased commercially: anti-β-Actin, (Sigma-Aldrich, Inc. St. Louise, MO); anti-P21/CIP1, (NeoMarker, Fremont, CA); anti-phosphorylated-p53 Ser¹⁵, total PARP, cleaved-PARP (Cell Signaling, Beverly, MA); enhanced chemifluorescence (ECF) detection kit, (Amersham Biosciences, Co. Piscataway, NJ); rabbit anti-P27KIP1, α-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA), Anti-PSA (DAKO, Glostrup, Denmark), anti-AR (BD PharMingen, San Diego, CA). SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA); HotStarTaq

Plus Master Mix Kit, RNeasy Midi kit (Qiagen Inc. Valencia, CA).

Cell culture and treatment. LNCaP cells were obtained from the American Type Culture Collection (Manassas, VA) and grown in RPMI 1640 supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) and 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, and 45 g/L glucose without antibiotics. When cells were ~50% confluent (usually 48 h after plating), the medium was changed and treatments with different compounds in different concentrations were started. Decursin and clinically used AR antagonist bicalutamide/Casodex served as positive controls for inhibition of cell growth and PSA expression (6,7). Non-transformed prostate epithelial RWPE-1 cells were purchased from ATCC and cultured per conditions recommended by ATCC.

In experiments where androgen stimulation was required, LNCaP cells were seeded in phenol red-free medium containing 5% charcoal-stripped serum (CCS; Atlanta Biologicals) to decrease background signaling. Androgen stimulation was provided by a non-metabolizable analogue mibolerone, which was a kind gift from Dr. Charles Young (Mayo Clinic, Rochester, MN).

Crystal Violet Staining. The cell number was estimated by first fixing the cells in 1% glutaraldehyde in PBS for 15 min and then by staining with 0.02% crystal violet solution (6,7). After extensive washing with distilled water, the plates were air dried and photographed. The retained crystal violet dye was dissolved in 70% ethanol and the optical absorbance was measured at 570 nm.

ELISA for PSA protein in conditioned medium. An assay kit from United Biotech, Inc. (Mountain View, CA) was used for measurement of PSA in conditioned medium (secreted) as described previously (6,13).

Analysis of cell cycle distribution. Treated and untreated LNCaP cells were dissociated with trypsin. The cells were stained with propidium iodide by using Krishan's method (14) and subjected to flow cytometry.

RNA Isolation and PCR. After LNCaP cells were treated with different compounds, total RNAs were isolated by using RNeasy Midi kit. Total RNA (4 µg) from each sample was reverse transcribed using Oligo-dT primers according to a reverse transcription Super Script™ II RT Kit manual (GIBCO-BRL, Foster City, CA). PCR was performed using HotStarTaq Master Mix Kit and a pair of oligonucleotide primers (Sigma-Genosys, The Woodlands, TX) as reported before (6). The PCR amplification started with 95°C for 15 min, and then preceded accordingly with conditions for different primers. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH): Forward 5'-TCA AGA AGG TGG TGA AGC AG-3', Reverse 5'-CTT ACT CCT TGG AGG CCA TG-3' (95°C 30 s, 57°C 1 min, 72°C 1 min, 25 cycles); AR: Forward 5'-ATG GAA GTG CAG TTA GGG-3', Reverse 5'-CAG GAT GTC TTT AAG GTC AGC-3' (95°C 30 s, 57°C 1 min, 72°C 1.5 min, 32 cycles); PSA: Forward: 5'-GAT GAC TCC AGC CAC GAC CT-3', Reverse: 5'-CAC AGA CAC CCC ATC CTA TC-3' (95°C 30 s, 57°C 1 min, 72°C 1.5 min, 22 cycles); 5α-Reductase-2: Forward: 5'-GAG GCT TAT TTG AAT ACG TAA C-3', Reverse: 5'-TTC TGA ACT TTG GAT ACT CTT C-3' (95°C 30 s, 58°C 1 min, 72°C 1.5 min, 38 cycles). PCR products were

fractionated in a 1.0% agarose gel containing ethidium bromide and visualized by UV illumination.

Western Blot. Cell lysates were prepared in RIPA buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 50 mM sodium fluoride 5 mM sodium orthovanadate, 1 mM DTT, 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride, 38 µg/ml aprotinin). The cell lysates were cleared by centrifugation at 4°C for 30 min. Protein concentration was determined by Lowry method. Protein lysate in RIPA buffer was denatured in SDS-PAGE sample buffer and subjected to SDS-PAGE on 7.5 or 12% gel. The proteins were transferred onto nitrocellulose membrane followed by blocking of membrane with 5% nonfat milk powder (w/v) in TBS (10 mM Tris, 100 mM NaCl, 0.1% Tween 20) for overnight at 4°C. The membranes were probed for the protein level using specific primary antibodies and visualized by alkaline phosphatase conjugated appropriate secondary antibodies and the substrate in ECF detection kit.

In experiments assessing effects of compounds on AR nuclear translocation, the cells were harvested and separated into nuclear and cytosolic fractions using NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Pierce, Rockford, IL) and the specificity of separation was confirmed by immunoblotting for total PARP and α-tubulin as the respective nuclear and cytosol markers.

Statistical analyses. Numerical data were expressed as mean ± SE when n ≥ 3. The data were analyzed by ANOVA followed by Bonferroni t test for pairwise multiple comparisons or other appropriate tests. *p* < 0.05 was considered statistically significant.

RESULTS

Growth inhibitory effect and PSA suppression activity of test compounds. To assess the overall growth inhibitory effects, we exposed LNCaP cells to 10 and 50 µM of each compound for 72 h in 6-well-plates. These doses were chosen to compare with the effective concentrations of a clinically used androgen antagonist drug, Casodex a.k.a. bicalutamide. The relative number of cells still remaining attached to the cell culture plasticware was estimated by crystal violet staining (Fig. 2A). For the sucrose-based compound #17, exposure at 50 µM led to a significant reduction (79%) of the adherent cells with an efficacy superior to Casodex (43%) (Fig. 2A) and similar to the growth inhibitory action of decursin (75%) (Fig. 2A). The other two sucrose compounds, #14 and #18, did not decrease LNCaP cell growth within the tested dose range.

Measurement of PSA by ELISA of the conditioned media from the above experiments showed that #17 exposure for 72 h led to a dramatic reduction of the secreted PSA (86% and 95% for 10 and 50 µM, respectively) (Fig. 2B), with overall inhibitory effect comparable to that of decursin (90%, 95%, for 10 and 50 µM, respectively). For the other two sucrose compounds, #14 and #18, a marginal decrease (32–36%) of secreted PSA was detected at the high test dose of 50 µM, but not at the low dose. These data support a structure–activity relationship (SAR) among the 3 sucrose

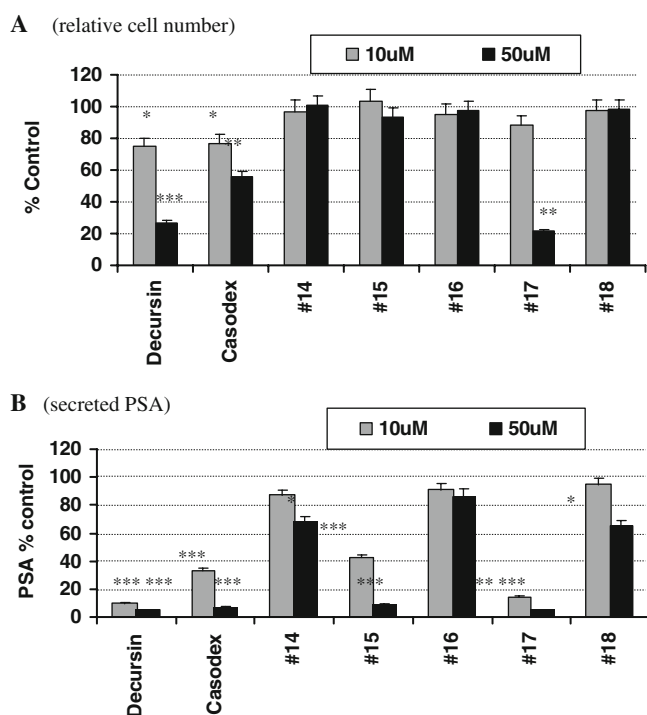


Fig. 2. Effects of test compounds on LNCaP cell number and secreted PSA after 72 h of exposure. **A** Cell number relative to DMSO-treated control was estimated by crystal violet staining. **B** Secreted PSA in conditioned medium. Cells were seeded in 6 well plates, duplicate wells per treatment. Decursin and Casodex were used for comparison of relative efficacy. The results of two experiments ($n=4$ wells) are presented. Mean \pm SEM. Statistical significance from control * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

compounds: three sinapoyl-moieties were required to suppress PSA expression and inhibit cell growth, whereas those with two sinapoyl-moieties were at least an order of magnitude less potent.

For the benzophenone compounds, **#15** and **#16**, exposure within the tested doses for 72 h did not affect the overall cell number (Fig. 2A). However, treatment with compound **#15** decreased secreted PSA in a dose-dependent manner (58%, 90%), and was similar in potency as Casodex (66%, 91%), but less effective than decursin (Fig. 2B). Compound **#16** was not effective at the highest concentration tested, i.e., 50 μ M (Fig. 2B). These data support a PSA-suppressing activity of compound **#15** in the absence of a growth inhibitory action.

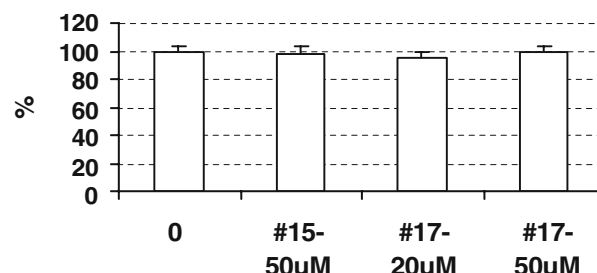
Compound **#17** did not affect the growth of non-transformed prostate epithelial cells but induced G_1 arrest in LNCaP cells. Interestingly and importantly, non-transformed prostate epithelial cells (RWPE-1) did not respond to the same dose range of exposure to compound **#17** after 3 days of treatment in terms of overall cell number (Fig. 3A). To further test whether cell cycle arrest contributed to and which phase of cell cycle was targeted for the selective growth inhibitory action of this compound against LNCaP cells, we treated exponentially growing cells in the complete medium containing 10% whole serum for 24 h before flow cytometry analyses. Compound **#17** treatment increased G_1 and decreased S phase cells in a concentration-dependent manner (ANOVA, $p<0.05$) without affecting the G_2 population (Fig. 3B), indicating a probable G_1 -arrest.

Effect of compound #15 and #17 on cellular PSA and AR protein. To determine whether the diminished level of secreted PSA by these two compounds was due to a reduction of cellular PSA protein and AR protein abundance, we next analyzed the cellular PSA and AR by Western blotting after 24 h exposure to 50 μ M of each compound. As shown in Fig. 4A, the secreted PSA was decreased by **#15** and **#17** in the same trend as for 72 h exposure (Fig. 2B). The cellular PSA protein level was decreased significantly by **#15** and **#17** (Fig. 4B). These two compounds also led to a significant decrease of AR protein abundance (Fig. 4B).

To further define the potency of these two compounds, we analyzed their effects at 10, 20 and 50 μ M after 24 h exposure (Fig. 4C). As low as 10 μ M of **#17** decreased cellular PSA and 20 μ M was needed to decrease AR protein level within this time frame of treatment. While each compound showed dose-dependency for decreasing both AR and PSA, **#15** was less potent than **#17**. Time course of AR and PSA changes showed that it took 6 h for PSA and 12 h for AR protein to show decreased abundance by **#17**, and slower still for **#15** (Fig. 4D), suggesting a possible involvement of transcriptional inhibition that took time to set in.

Compound #17 activated p53 tumor suppressor protein and induced apoptosis. To determine whether the PSA and

A (RWPE-1 Cell #, 3 Days)



B (LNCaP, 24 h)

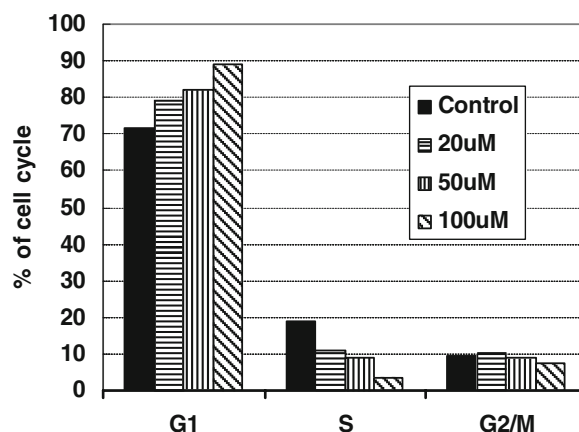


Fig. 3. Selective growth inhibitory activity of compound **#17** on LNCaP cells. **A** Lack of growth inhibitory effect of **#15** and **#17** on non-transformed RWPE-1 human prostate epithelial cells after 72 h treatment. Each compound and concentration was tested in triplicate wells of 12-well plates. ANOVA, $p>0.5$. **B** Flow cytometric analyses of LNCaP cell cycle distribution at 24 h after treatment with compound **#17**. Each column represents the average of two T₂₅ flasks, variation within 2%. ANOVA $p<0.05$ for G_1 and S phase distribution changes due to treatment with **#17**.

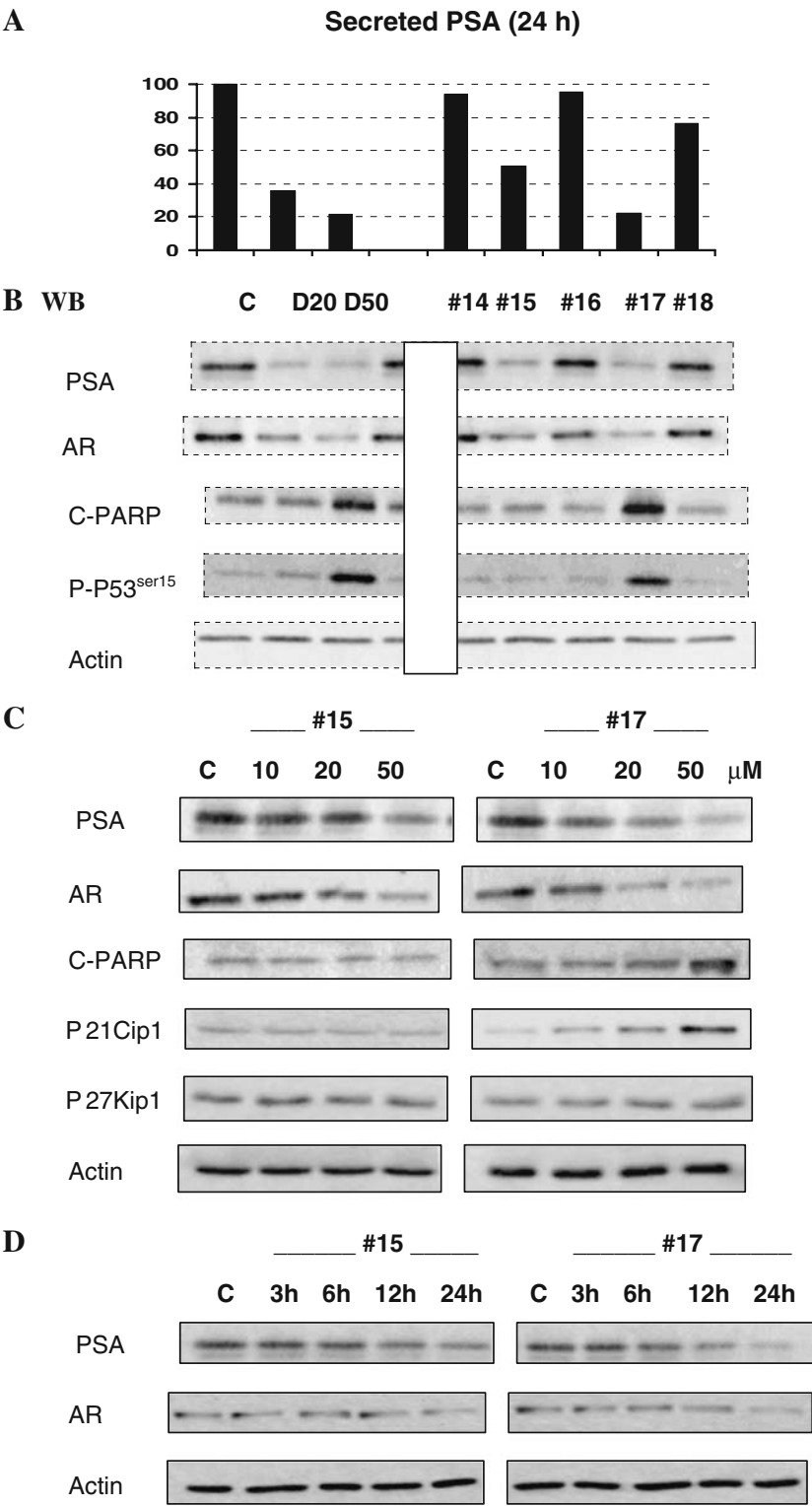


Fig. 4. Effects of test compounds on cellular PSA, AR and apoptosis-related proteins in LNCaP cells. Decursin was used for comparison (D20, D50 signify decursin dosing concentration of 20 and 50 μ M). **A** ELISA measurement of secreted PSA at 24 h of exposure to 50 μ M of test compound. Each column represents the average of duplicate assays of the condition medium from a single T₂₅ flask. **B** Western blot analyses of cellular PSA, AR and cleaved PARP and phosphorylated P53 on Ser¹⁵ as biomarkers of apoptosis. **C** Dose-response patterns of PSA and AR in relationship to PARP cleavage and induction of P53-target gene product P21Cip1 to **#15** and **#17** exposure for 24 h. **D** Time course of PSA and AR protein down regulation by **#15** and **#17** at 50 μ M exposure concentration.

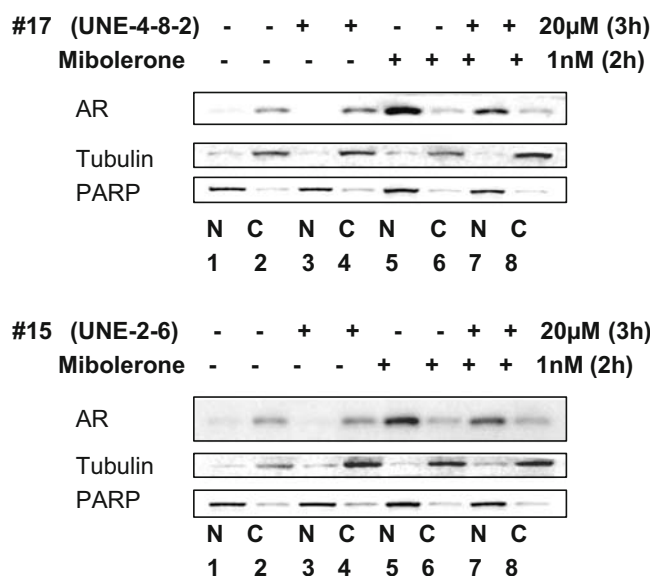


Fig. 5. Effect of compound **#15** and **#17** on androgen-stimulated cytosol-to-nuclear translocation of AR in LNCaP cells. LNCaP cells were grown in 5% charcoal stripped serum medium for 2 days to decrease background signaling. The cells were pretreated with 20 μM of compound **#15** and **#17** for 1 h and were stimulated with mibolerone (1 nM) for 2 h in the continued presence of each compound. Nuclear (N) and cytosolic (C) fractions were prepared for immunoblot analyses for AR. PARP and α-tubulin were detected to show the specificity of the nuclear and cytosol preparations.

AR suppressing actions were dissociable from apoptosis, we analyzed apoptotic cleavage of PARP, which is a canonical protein substrate for caspase-3, after 24 h exposure (Fig. 4B). Compound **#17** increased cleavage of PARP, which was accompanied by an increased p53 Ser¹⁵ phosphorylation, with similar potency as decursin (Fig. 4B). On the other hand, **#15** did not cause cleavage of PARP (Fig. 4B) or morphologically observable cell death (data not shown).

In the dose-response experiment (Fig. 4C), the cellular PSA was decreased by as low as 10 μM of compound **#17** without an increased cleavage of PARP, which was detectable at 50 μM. The p53 transcriptional target P21Cip1 was increased in LNCaP cells treated with **#17**, whereas there was no change of P27Kip1 (Fig. 4C). The data support the inhibition of AR-PSA signaling by **#17** at lower concentrations than that was required to induce pro-apoptotic action in LNCaP cells. The apoptotic PARP cleavage induced by this compound was associated with an activation of p53 Ser¹⁵ phosphorylation and P21Cip1 induction. Being a critical G₁ CDK inhibitor (15), P21Cip1 induction might contribute to and mediate the G₁ arrest observed in Fig. 3B.

Effect of compound #15 and #17 on AR cytosol to nuclear translocation. Upon androgen binding, the cytosol AR in androgen responsive cells will undergo conformational change, dimerize and translocate from the cytosol (C) to the nucleus (N) to activate target gene transcription. To determine whether **#15** and **#17** affected androgen-stimulated AR nuclear translocation, we grew LNCaP cells in phenol red-free medium supplemented with 5% charcoal-stripped serum (CSS) for 2 days to decrease basal signaling. The cells were pretreated

with 20 μM of **#15** and **#17** for 1 h and were stimulated with mibolerone (1 nM) for 2 h in the continued presence of each compound. Nuclear and cytosolic fractions were prepared for immunoblot analyses. As shown in Fig. 5, PARP and α-tubulin detection showed acceptable specificity of the nuclear and cytosol preparations. Mibolerone stimulation converted a predominantly cytosolic distribution pattern for AR under androgen-deprived state (Fig. 5, lanes 1 and 2) to one that was mostly nuclear localized (lanes 5 and 6). Compound **#15** and **#17** decreased nuclear AR level (compare lanes 7 with lane 5). These results indicate that **#15** and **#17** rapidly inhibited androgen-stimulated AR nuclear translocation in addition to decreasing AR protein abundance later.

Effect of compound #15 and #17 on the mRNA level of AR and PSA. To determine whether the inhibitory action of **#15** and **#17** on AR and PSA occurred at the transcript level, we examined the level of AR and PSA mRNA after exposure for 24 h (Fig. 6). As a reference, we included decursin, which we had shown to decrease PSA mRNA level but not AR mRNA level in LNCaP cells (6,7), to validate the reverse transcription-PCR (RT-PCR) detection methodology (Fig. 6, lane 3). The RT-PCR assay detected a significant reduction of PSA mRNA abundance at 24 h of treatment with **#15** and **#17** (Fig. 6, lanes 4 and 5), with the extent of reduction in excellent agreement with the decreased levels of secreted PSA detected by ELISA.

In contrast to the lack of any effect of decursin on the AR mRNA level (lane 3 vs. 2), compound **#15** and **#17** decreased AR mRNA in moderate extent (Fig. 6, lanes 4 and 5 vs. 2). Furthermore, the mRNA for 5α-reductase-2 gene was increased by compound **#17** (lane 5), but not by decursin or **#15** (Fig. 6, lanes 3 and 4), indicating a possible enhancement of the ability of LNCaP cells treated with **#17** to convert T to DHT. We should note that in future work we need to determine whether the 5α-reductase-2 mRNA change induced by **#17** indeed translates to protein/enzyme functional differences. If such is found, the overall anti-AR signaling activity of **#17** could be determined by the balance of these opposing mechanisms. Taken together, the results indicate that the suppressing action of **#15** and **#17** on PSA protein level was largely mediated by decreasing PSA mRNA transcript abundance, which was accompanied by decreased AR mRNA and protein levels and AR nuclear exclusion.

DISCUSSION

Androgen/hormonal-ablation therapies are standard treatments for even advanced metastatic PCa, because most of the cancer cells are still androgen-responsive for their survival (16). In hormone-refractory cancer that inevitably arises after the failure of hormonal ablation therapies, which usually bring about 1.5–2 years of disease remission, most of the PCa cells still retain wild type AR (2,3,17). In about a third of patients with androgen-refractory prostate cancer, there is an amplification of the AR gene, which is not present when the tumors are hormone-dependent (18). This gene amplification leads to an increase in the expression of the AR protein and enhanced activation by low levels of androgens. The AR expressed in androgen-independent prostate cancer

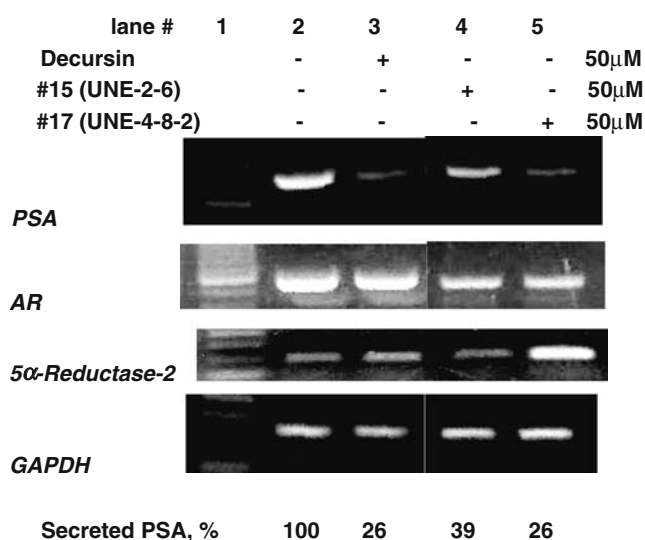


Fig. 6. RT-PCR analyses of the effects of compound **#15** and **#17** and decursin on the steady state mRNA level of PSA, AR and 5 α -reductase-2 after 24 h treatment of LNCaP cells. The level of secreted PSA in conditioned medium was given below each corresponding lane.

also appears to be transcriptionally active because these tumors express high levels of multiple genes that are normally regulated by AR, including PSA and prostate-specific membrane antigen (2,3). Indeed, ectopic over-expression of the wild-type AR can be a molecular determinant of hormone refractoriness and may turn the best-characterized and clinically used AR antagonist drug Casodex/bicalutamide into an agonist (19). On the other hand, experimental approaches that suppress AR protein/function using anti-AR ribozyme or inactivating monoclonal antibodies (20,21) or a decoy oligonucleotide containing an androgen responsive element to sequester endogenous AR (22) support the critical role of AR in PCa cell survival and proliferation in even the androgen-independent stage. More recent work using siRNA to knockdown AR further confirms these findings (23). Furthermore, PSA protein has been convincingly shown to contribute to/mediate the AR-mediated PCa cell growth *in vitro* and *in vivo* (24). These findings suggest that the AR-PSA signaling axis plays a critical role in the development of androgen-dependent as well as androgen-refractory PCa and provides attractive targets for PCa chemoprevention and treatment by natural products/compounds, especially those with mechanisms different from existing androgen antagonist drugs (e.g., Casodex) or 5 α -reductase inhibitors (e.g., finasteride).

We presented data that among the 3 sucrose compounds tested, **#17** (UNE-4-8-2), beta-D-(3,4-di-sinapoyl)fructofuranosyl-alpha-D-(6-sinapoyl)glucopyranoside with three sinapoyl-moieties was at least an order of magnitude more potent at decreasing PSA and AR expression than those with only two sinapoyl-moieties: **#18** (UNE-7-4), beta-D-(3-sinapoyl)fructofuranosyl-alpha-D-(6-sinapoyl)glucopyranoside, **#14** (UNB-2), alpha-D-(3-sinapoyl)fructofuranosyl-alpha-D-(6-sinapoyl)glucopyranoside (Figs. 2B and 4). Compound **#17** also was more potent at inducing G₁ arrest (Fig. 3B), P53 Ser¹⁵ phosphorylative activation and apoptotic PARP

cleavage (Fig. 4) and to decrease overall cell growth (Fig. 2) than those with only two sinapoyl-moieties. Therefore, these comparisons afforded a SAR for the minimum of three sinapoyl-moieties attached to sucrose to suppress AR and PSA protein expression, and inhibit cell growth and to induce p53-activation and caspase-mediated apoptosis. More importantly, **#17** induced G₁ arrest in cancer cells but did not inhibit the growth of RWPE-1 non-transformed prostate epithelial cells (Fig. 3A), supporting a preferential selectivity against neoplastic cells.

Mechanistic investigations revealed that compound **#17** might inhibit AR-PSA signaling at multiple levels: It inhibited androgen-stimulated AR translocation from the cytosol to the nucleus (Fig. 5), decreased AR protein abundance (Fig. 4), and decreased AR mRNA level (Fig. 6). These effects on the AR might in part account for the decreased PSA mRNA level through reduced trans-activation of PSA gene transcription (Fig. 6) and decreased cellular PSA expression (Fig. 4) and diminished PSA secretion into the medium (Figs. 2B and 4A). A dose-response experiment indicated that the changes of AR-PSA signaling could be observed at a dosage of **#17** that did not activate p53 phosphorylation or apoptotic PARP cleavage (Fig. 4C). Such observations support a notion that the anti-AR signaling actions were not the consequence of cellular apoptosis, rather on the contrary, they might causally contribute to the cell cycle arrest and even cellular demise.

Interestingly, structural differences notwithstanding (Fig. 1), compound **#17** exerted anti-AR signaling action sharing remarkable similarities in the cellular processes and molecular "targets" with decursin, such that both induce AR protein down regulation (Fig. 4 and refs. (6,7), both inhibit AR cytosol-to-nuclear translocation (Fig. 5 and refs. (6,7) and activate p53 phosphorylation and apoptosis (Fig. 4, refs. (6,7); These actions differed from Casodex at similar dose range tested, which could act as a partial agonist in the absence of androgen (6,7). Yet compound **#17** contrasted with decursin in terms of the differential impact on AR mRNA level (Fig. 6) and the 5 α -reductase-2 mRNA (Fig. 6). In future work we need to determine whether the 5 α -reductase 2 mRNA change induced by **#17** indeed translates to protein/enzyme functional differences. If such is found, this feature may limit its overall anti-AR signaling activity and adds a cautionary note to its utility as an anti-AR agent.

The modes of action of **#15** in the dose range tested, on the other hand, support it as an anti-AR signaling agent in the absence of cell growth inhibition or apoptosis. The *in vitro* potency of compound **#15** to inhibit AR-PSA signaling was similar to Casodex, but weaker than **#17** or decursin. The ability of **#15** to induce AR down regulation at both the mRNA (Fig. 6) and protein levels (Fig. 4) differed from Casodex at similar dose range, which did not decrease these parameters (6,7). Higher dosages of **#15** may be required to exert an observable impact on the cell growth and survival in our model system.

In terms of the potential for pharmaceutical development, we chose to evaluate the herbal compounds at dosages comparable to the effective concentration range of clinically used anti-androgen drug Casodex. Since compounds **#17** and **#15** have better or comparable anti-AR signaling activity than Casodex *in vitro*, the potential limitations on their use as therapeutic agents could be poor *in vivo* bioavailability and

stability and unfavorable pharmacokinetics. Due to the limited amount of material available to us, we could not address these issues in the current work. Animal studies are warranted to establish the *in vivo* anti-AR signaling potency of these compounds and to judge the merit for further development for PCa chemoprevention or therapy. If future work establishes favorable *in vivo* attributes of **#17** and/or **#15**, it is possible that these compounds could be used as monotherapy individually or combined with existing androgen ablation drugs (e.g., Casodex, finasteride) to achieve synergistic suppressing effects on AR signaling with lower levels of each and possibly decrease their side effects.

ACKNOWLEDGMENT

This work was supported in part by The Hormel Foundation, Prostate Cancer Foundation, by MRC grant R13-2007-019-00000-0 from Korea Ministry of Education, Science and Technology. The authors thank Todd Schuster for performing flow cytometry analyses.

Conflict of interest statements All authors have no personal or financial conflict of interest and have not entered into any agreement that could interfere with our access to the data on the research or on our ability to analyze the data independently, to prepare manuscripts and to publish them.

REFERENCES

1. A. Jemal, R. Siegel, E. Ward et al. Cancer statistics, 2008. *CA Cancer J. Clin.* **58**(2):71–96 (2008) Mar–Apr. doi:10.3322/CA.2007.0010.
2. M. E. Taplin, and S. P. Balk. Androgen receptor: a key molecule in the progression of prostate cancer to hormone independence. *J. Cell Biochem.* **91**(3):483–490 (2004) Feb 15. doi:10.1002/jcb.10653.
3. C. A. Heinlein, and C. Chang. Androgen receptor in prostate cancer. *Endocr. Rev.* **25**(2):276–308 (2004) Apr. doi:10.1210/er.2002-0032.
4. G. Andriole, N. Bruchovsky, L. W. Chung et al. Dihydrotestosterone and the prostate: the scientific rationale for 5 α -reductase inhibitors in the treatment of benign prostatic hyperplasia. *J. Urol.* **172**(4 Pt 1):1399–1403 (2004) Oct. doi:10.1097/01.ju.0000139539.94828.29.
5. I. M. Thompson, P. J. Goodman, C. M. Tangen et al. The influence of finasteride on the development of prostate cancer. *N. Engl. J. Med.* **349**(3):215–224 (2003) Jul 17. doi:10.1056/NEJMoa030660.
6. C. Jiang, H. J. Lee, G. X. Li et al.. Potent antiandrogen and androgen receptor activities of an Angelica gigas-containing herbal formulation: identification of decursin as a novel and active compound with implications for prevention and treatment of prostate cancer. *Cancer Res.* **66**(1):453–463 (2006) Jan 1. doi:10.1158/0008-5472.CAN-05-1865.
7. J. Guo, C. Jiang, Z. Wang et al. A novel class of pyranocoumarin anti-androgen receptor signaling compounds. *Mol. Cancer Ther.* **6**(3):907–917 (2007) Mar. doi:10.1158/1535-7163.MCT-06-0231.
8. M. C. Song, F. Nigussie, H. J. Yang, H. H. Kim, J. Y. Kim, D. K. Chung, and N. I. Baek. Phenolic glycosides from *Lindera fruticosa* root and their inhibitory activity on osteoclast differentiation. *Chem. Pharm. Bull. (Tokyo)*. **56**(5):707–710 (2008) May. doi:10.1248/cpb.56.707.
9. M. C. Song, F. Nigussie, T. S. Jeong, C. Y. Lee, F. Regassa, T. Markos, and N. I. Baek. Phenolic compounds from the roots of *Lindera fruticosa*. *J. Nat. Prod.* **69**(5):853–855 (2006). May. doi:10.1021/np060048b.
10. C. Costa, A. Bertazzo, G. Allegri, O. Curcuruto, and P. Traldi. Indole Alkaloids from the Roots of an African Plant *Securidaca Longipedunculata*. I. Isolation by column chromatography and preliminary structural characterization by mass spectrometry. *J. Heterocyc. Chem.* **29**:1641–1647 (1992).
11. N. de Tommasi, S. Piacente, F. de Suione, and C. Pizza. New sucrose derivatives from the bark of *Securidaca longipedunculata*. *J. Nat. Prod.* **56**(1):134–137 (1993). doi:10.1021/np50091a020.
12. W.-Y. Kang, Z.-M. Wang, Z.-Q. Li, and X.-J. Xu. Three New Compounds from *Securidaca inappendiculata*. *Helvetica Chim. Acta.* **88**:2771–2776 (2005). doi:10.1002/hlca.200590217.
13. S. D. Cho, C. Jiang, B. Malewicz et al. Methyl selenium metabolites decrease prostate-specific antigen expression by inducing protein degradation and suppressing androgen-stimulated transcription. *Mol. Cancer Ther.* **3**:605–611 (2004).
14. A. Krishan. Rapid flow cytofluorometric analysis of mammalian cell cycle by propidium iodide staining. *J. Cell Biol.* **66**:188–193 (1975). doi:10.1083/jcb.66.1.188.
15. C. J. Sherr, and J. M. Roberts. CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev.* **13**:1501–1512 (1999). doi:10.1101/gad.13.12.1501.
16. H. Miyamoto, E. M. Messing, and C. Chang. Androgen deprivation therapy for prostate cancer: current status and future prospects. *Prostate.* **61**:332–353 (2004). doi:10.1002/pros.20115.
17. B. J. Feldman, and D. Feldman. The development of androgen-independent prostate cancer. *Nat. Rev. Cancer.* **1**:34–45 (2001). doi:10.1038/35094009.
18. P. Koivisto, J. Kononen, C. Palmberg, T. Tammela, E. Hyytinen, J. Isola et al. Androgen receptor gene amplification: a possible molecular mechanism for androgen deprivation therapy failure in prostate cancer. *Cancer Res.* **57**:314–319 (1997).
19. C. D. Chen, D. S. Welsbie, C. Tran et al. Molecular determinants of resistance to antiandrogen therapy. *Nat. Med.* **10**:33–39 (2004). doi:10.1038/nm972.
20. S. Chen, C. S. Song, Y. Lavrovsky et al. Catalytic cleavage of the androgen receptor messenger RNA and functional inhibition of androgen receptor activity by a hammerhead ribozyme. *Mol. Endocrinol. (Baltimore, Md.)*. **12**(10):1558–1566 (1998)Oct.
21. O. L. Zegarar-Moro, L. J. Schmidt, H. Huang, and D. J. Tindall. Disruption of androgen receptor function inhibits proliferation of androgen-refractory prostate cancer cells. *Cancer Res.* **62**(4):1008–1013 (2002)Feb 15.
22. K. Kuratsukuri, K. Sugimura, K. Harimoto, H. Kawashima, and T. Kishimoto. “Decoy” of androgen-responsive element induces apoptosis in LNCaP cells. *Prostate.* **41**(2):121–126 (1999) Oct 1. doi:10.1002/(SICI)1097-0045(19991001)41:2<121::AID-PROS6>3.0.CO;2-Q.
23. X. Liao, S. Tang, J. B. Thrasher, T. L. Griebing, and B. Li. Small-interfering RNA-induced androgen receptor silencing leads to apoptotic cell death in prostate cancer. *Mol. Cancer Ther.* **4**(4):505–515 (2005) Apr. doi:10.1158/1535-7163.MCT-04-0313.
24. Y. Niu, S. Yeh, H. Miyamoto et al. Tissue prostate-specific antigen facilitates refractory prostate tumor progression via enhancing ARA70-regulated androgen receptor transactivation. *Cancer Res.* **68**(17):7110–7119 (2008) Sep 1. doi:10.1158/0008-5472.CAN-07-6507.