

ORIGINAL ARTICLE

Isolation and structure elucidation of a novel androgen antagonist, arabilin, produced by *Streptomyces* sp. MK756-CF1

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In the course of screening for a new type of androgen receptor (AR) antagonist, we isolated a novel compound, arabilin, with two structural isomers, spectinabilin and SNF4435C, produced by *Streptomyces* sp. MK756-CF1. Structure elucidation on the basis of the spectroscopic properties showed that arabilin is a novel polypropionate-derived metabolite with a *p*-nitrophenyl group and a substituted γ -pyrone ring. Arabilin competitively blocked the binding of androgen to the ligand-binding domain of AR *in vitro*. In addition, arabilin inhibited androgen-induced prostate-specific antigen mRNA expression in prostate cancer LNCaP cells.

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INTRODUCTION

Androgen receptor (AR), a member of the nuclear receptor superfamily, is a critical mediator of prostate cancer; therefore, treatment with AR antagonists is expected to be an effective prostate cancer therapy. AR antagonists can be classified into two structural types, steroidal and nonsteroidal compounds.^{1,2} Steroidal AR antagonists often exhibit side effects because of cross-reactivity with other steroid hormone nuclear receptors, such as estrogen receptor and progesterone receptor. On the other hand, anilide-type compounds, such as flutamide and bicalutamide, are representative of nonsteroidal AR antagonists. Although these anilide-type AR antagonists have been clinically used for prostate cancer therapy, prostate cancer often advances to a hormone-refractory state after long-term treatment with AR antagonists.³ The mutation in AR is considered a possible reason for rendering prostate cancer cells hormone refractory.⁴ Furthermore, anilide-type AR antagonists act as agonists toward hormone-refractory prostate cancer cells in some cases.^{5–7} Thus, development of a new type of AR antagonist is an attractive strategy to overcome prostate cancers that are resistant to the known AR antagonists.

In the course of screening for a new type of AR antagonist, we isolated a novel compound, arabilin, with two known structural isomers, spectinabilin and SNF4435C, from *Streptomyces* sp. MK756-CF1. In this paper, the isolation, structure elucidation and biological activities of arabilin are reported.

RESULTS AND DISCUSSION

Screening for binding inhibitors of DHT and AR

To obtain a new type of AR antagonist with a nonsteroidal/nonanilide-type structure, we first screened more than 2000 microbial extracts to find inhibitors, which could inhibit the binding of dihydrotestosterone (DHT) to AR using a [³H]DHT-AR *in vitro* binding assay. In the course of screening, we found that the culture broth extract of strain MK756-CF1 inhibited the binding of DHT to AR.

Taxonomy of the producing strain

Strain MK756-CF1 produced spore chains on aerial mycelia, which developed from branched substrate mycelia. The partial gene sequence (1412 bp) coding 16S ribosomal RNA of MK756-CF1 showed high homology with those of members of the genus *Streptomyces*, such as *Streptomyces spectabilis* (National Institute of Technology and Evaluation Biological Research Center (NBRC) 13423T 1408/1413 bp, 99%) and *Streptomyces flavofungini* (NBRC 13371T 1391/1412 bp, 98%). These phenotypic and genotypic properties suggested that strain MK756-CF1 belonged to the genus *Streptomyces*. Further detailed taxonomic study of strain MK756-CF1 is in progress.

Isolation of arabilin, spectinabilin and SNF4435C

The cultivation of strain MK756-CF1 was carried out in 60 500-ml Erlenmeyer flasks containing pressed wheat (2.4 kg) because this solid-state fermentation enabled the strain to produce abundant active

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components. After fermentation, the culture was extracted with EtOH (21), filtrated and concentrated *in vacuo*. This suspension was adjusted to pH 7.0, followed by extraction with EtOAc (31) twice, and the organic layer was concentrated to give a pink oily residue (2.2 g). Thus, the obtained crude active oil was subsequently subjected to silica gel column chromatography (Silica gel 60, 60–230 μm ; Merck, Darmstadt, Germany) using an *n*-hexane-EtOAc stepwise system. One active fraction (*n*-hexane-EtOAc, 2:1) was further purified by preparative octadecyl silyl (ODS) HPLC (Sun Fire, 10 μm , 19 \times 250 mm; Waters, Milford, MA, USA) with 80% aqueous MeOH to give a pure novel compound, arabilin (3.3 mg) (Figure 1). Another active fraction obtained by silica gel column chromatography (*n*-hexane-EtOAc, 1:1) was also further purified by preparative ODS HPLC to give spectinabilin (3.0 mg)⁸ and SNF4435C (6.0 mg)⁹ (Figure 1). Spectinabilin and SNF4435C were reported as a weak inhibitor of Rauscher leukemia virus reverse transcriptase⁸ and a potent immunosuppressant,⁹ respectively.

Structure elucidation of arabilin

The physicochemical properties of arabilin, as well as spectinabilin and SNF4435C, are summarized in Table 1.^{8,10} From HRESI-MS measurements in combination with ¹H and ¹³C NMR data, the molecular formula of arabilin was determined to be C₂₈H₃₁NO₆ (found: 478.2215 [M+H]⁺, calcd: 478.2224), the same as spectinabilin and SNF4435C. As the ¹H and ¹³C NMR spectra of arabilin were partially similar to those of spectinabilin,^{8,11} a structural study of arabilin was performed by comparing with spectinabilin. The IR spectrum revealed that arabilin possesses a ketone conjugated with a double bond (1666 cm⁻¹) and a nitro group (1516 and 1342 cm⁻¹), as does spectinabilin. On the other hand, the UV spectrum of arabilin (λ_{max} : 263 nm (ϵ 18 400), 315 nm (sh, ϵ 10 300)) was different from that of spectinabilin (λ_{max} : 252 nm (ϵ 17 600), 268 nm (ϵ 18 200), 367 nm (ϵ 15 500)).⁸ The structure of arabilin was mainly determined by NMR spectral analyses as follows. We established direct connectivity

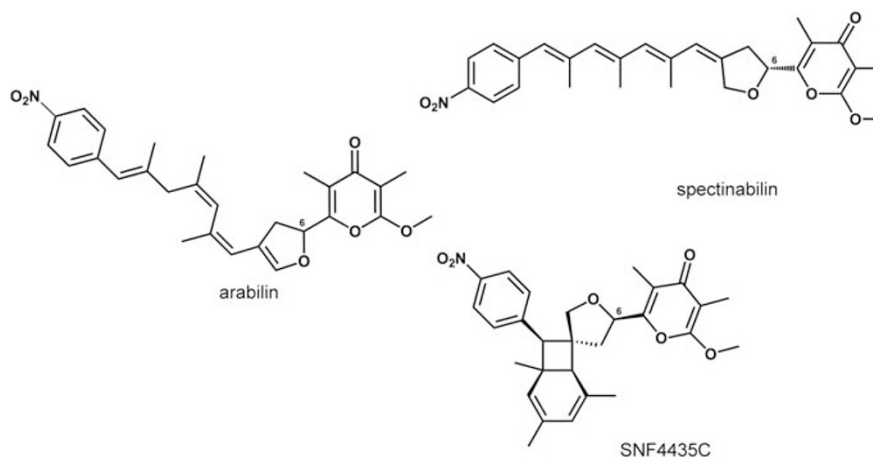


Figure 1 Structures of arabilin, spectinabilin and SNF4435C.

Table 1 Physicochemical properties of arabilin, spectinabilin and SNF4435C

	<i>Arabilin</i>	<i>Spectinabilin</i>	<i>SNF4435C</i>
Appearance	Pale yellow powder	Pale yellow powder	Pale yellow powder
Molecular formula	C ₂₈ H ₃₁ NO ₆	C ₂₈ H ₃₁ NO ₆	C ₂₈ H ₃₁ NO ₆
Molecular weight	477	477	477
HRESI-MS (<i>m/z</i> , Positive)			
Calcd	478.2224 (as C ₂₈ H ₃₂ NO ₆)	—	—
Found	478.2215	—	—
Optical rotation [α] _D	−166.2° (<i>c</i> 0.13, CHCl ₃ , 25°C)	+60.6° ^a (<i>c</i> 5.0, CHCl ₃ , 26°C)	−105.6° ^b (<i>c</i> 0.1, CHCl ₃ , 26°C)
IR ν_{max} (cm ⁻¹) (KBr)	2956, 2854, 1666, 1597, 1516, 1342	1670, 1520, 1340 ^a	2950, 2850, 1695, 1600, 1520, 1350 ^b
UV λ_{max} (nm) (ϵ)	263 (18400), 315 (sh, 10300) (MeOH)	218 (19100), 252 (17600), 268 (18200), 367 (15500) (EtOH) ^a	271 (19300) (MeOH) ^b
TLC (R_f) ^c	0.68	0.51	0.55
HPLC (Rt, min) ^d	25.2 (85% MeOH)	23.5 (85% MeOH)	16.7 (85% MeOH)
Solubility			
Soluble	CHCl ₃ , MeOH	CHCl ₃ , MeOH	CHCl ₃ , MeOH
Insoluble	<i>n</i> -hexane, H ₂ O	<i>n</i> -hexane, H ₂ O	<i>n</i> -hexane, H ₂ O

^aKakinuma *et al.*⁸

^bTakahashi K. *et al.*¹⁰

^cSilica gel TLC (Kieselgel 60F₂₅₄; Merck); mobile phase, *n*-hexane-EtOAc (1:2).

^dColumn, SunFire ODS (Waters, 5 μm , 4.6 \times 250 mm); mobile phase, aqMeOH; flow rate, 0.7 ml min⁻¹.

between each proton and carbon by the heteronuclear multiple quantum coherence (HMQC) spectrum; the ^1H and ^{13}C spectral data for arabilin are shown in Table 2. The ^1H - ^1H COSY and Heteronuclear Multiple Bond Coherence (HMBC) spectra proved that arabilin has both a 2-methoxy-3,5-dimethyl- γ -pyrone moiety

Table 2 ^{13}C - and ^1H -NMR data for arabilin in CDCl_3

Number	δ_{C} (p.p.m.)	δ_{H} (p.p.m.)
1	162.1	—
1a	55.3	3.92 (3H, s)
2	99.9	—
2a	6.9	1.86 (3H, s)
3	180.6	—
4	119.6	—
4a	9.4	2.02 (3H, s)
5	154.2	—
6	77.2	5.59 (1H, dd, $J=7.7, 11.0$)
7	35.6	2.85 (1H, dd, $J=7.5, 15.2$) 3.08 (1H, dd, $J=11.0, 15.2$)
8	114.9	—
8a	144.4	6.48 (1H, s)
9	118.5	5.91 (1H, s)
10	131.7	—
10a	25.3	1.88 (3H, bs)
11	128.6	5.99 (1H, s)
12	134.7	—
12a	22.8	1.73 (3H, bd)
13	44.2	2.91 (2H, s)
14	141.1	—
14a	18.1	1.81 (3H, bd)
15	124.9	6.32 (1H, s)
16	145.1	—
17	129.3	7.35 (2H, d, $J=8.8$)
18	123.5	8.17 (2H, d, $J=8.8$)
19	145.9	—

Chemical shifts in p.p.m. from TMS as an internal standard.

(C-1 to C-5) and a *p*-nitrophenyl group (C-16 to C-19), as does spectinabilin (Figure 2 and Jacobsen *et al.*¹¹). This finding and the difference between the UV spectrum of arabilin and that of spectinabilin imply that the tetraene moiety combined with a substituted furan moiety in spectinabilin is not preserved in arabilin. In the ^1H NMR spectra, one singlet methylene signal (δ_{H} 2.91, H-13, 2H) was observed only in arabilin (Table 2 and Jacobsen *et al.*¹¹). In the HMBC spectrum of arabilin, ^1H - ^{13}C long-range couplings from two methyl protons (δ_{H} 1.73, H-12a and δ_{H} 1.81, H-14a) to an sp^3 carbon (δ_{C} 44.2, C-13) were observed (Figure 2), whereas no ^1H - ^{13}C long-range coupling from the methyl proton to sp^3 carbon was observed in that of spectinabilin (data not shown). In addition, ^1H - ^{13}C long-range couplings from a methine proton (δ_{H} 6.48, H-8a) to a methine carbon bearing oxygen (δ_{C} 77.2, C-6), a methylene carbon (δ_{C} 35.6, C-7) and a quaternary sp^2 carbon (δ_{C} 114.9, C-8) indicated that C-8 and C-8a are connected by a double bond in arabilin but not in spectinabilin. Thus, the partial structures of arabilin other than a substituted γ -pyrone ring and a *p*-nitrophenyl group (C-5 to C-16) were also determined on the basis of ^1H - ^1H COSY and HMBC analyses (Figure 2). The geometries of C-8/C-8a, C-9/C-10, C-11/C-12 and C-14/C-15 were determined to be *E*, *Z*, *Z* and *E* by NOE observation between H-8a (δ_{H} 6.48) and H-9 (δ_{H} 5.91), H-9 and H-10a (δ_{H} 1.88), H-11 (δ_{H} 5.99) and H-12a (δ_{H} 1.73), and H-13 (δ_{H} 2.91) and H-15 (δ_{H} 6.32), respectively (Figure 2). From the above findings, the planar structure of arabilin was determined as shown in Figure 1. Thus, it was revealed that all arabilin and its structural isomers, spectinabilin and SNF4435C, had a *p*-nitrophenyl group and a substituted γ -pyrone ring. The configurations at C-6 in spectinabilin, its analog aureothin and SNF4435C were determined as *R*.^{12,13} The stereochemistry of arabilin has not yet been determined and is now under study.

Effects of arabilin, spectinabilin and SNF4435C on binding of DHT to AR

Arabilin, spectinabilin and SNF4435C inhibited the binding of DHT to AR in a dose-dependent manner (Figure 3). The IC_{50} values of arabilin, spectinabilin and SNF4435C were $11\ \mu\text{M}$, $13\ \mu\text{M}$ and $7\ \mu\text{M}$, respectively, and these inhibitory activities were about 10-fold more

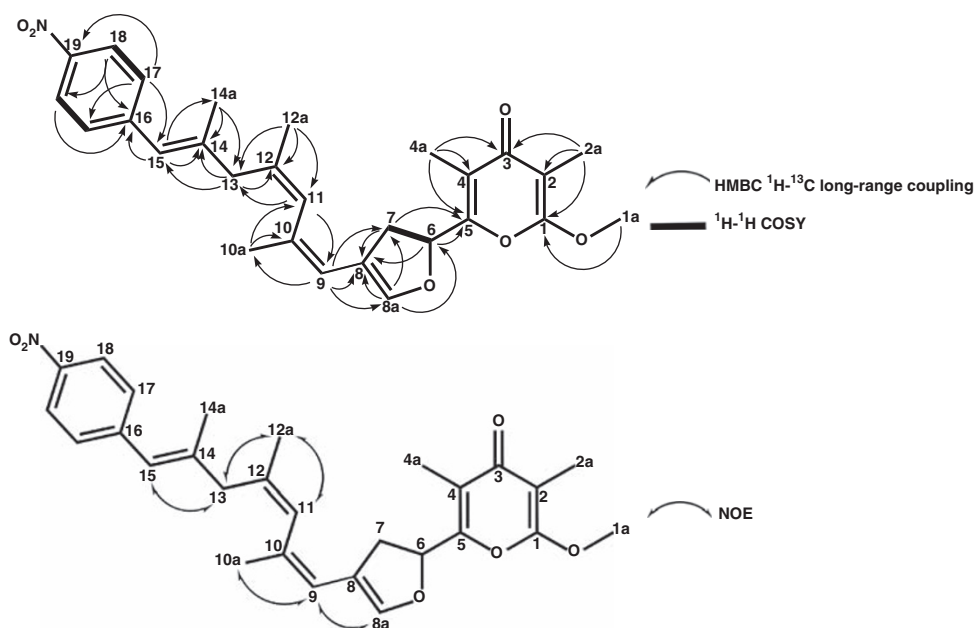


Figure 2 Structures of arabilin elucidated by ^1H - ^1H COSY, NOE and heteronuclear multiple bond coherence (HMBC) experiments.

potent than that of flutamide, which was clinically used for the treatment for prostatic diseases. On the other hand, arabilin, spectinabilin and SNF4435C did not show inhibitory activity against binding of estradiol to estrogen receptor up to 100 μM (data not shown).

Effects of arabilin, spectinabilin and SNF4435C on DHT-induced PSA expression

Prostate-specific antigen (PSA) is a 33-kDa serine protease, whose expression in the prostate is triggered by androgen-mediated action of AR; therefore, to determine whether arabilin, spectinabilin and

SNF4435C showed AR antagonistic activity, we examined the effects of these compounds on DHT-induced expression of PSA mRNA in prostate cancer LNCaP cells. As shown in Figure 4, arabilin, spectinabilin and SNF4435C inhibited the DHT-induced expression of endogenous PSA mRNA in LNCaP cells with IC_{50} values of 210 nM, 1.75 nM and 274 nM, respectively, whereas they did not induce the expression of PSA mRNA (data not shown). These three compounds did not inhibit DHT-independent GAPDH gene expression under these conditions (data not shown). Spectinabilin was reported to inhibit *Ascaris* NADH-fumarate reductase,¹⁴ and we found that it inhibited mitochondrial respiration of tumor cells, but its IC_{50} value is about 100 nM. Therefore, inhibition of DHT-induced expression of PSA mRNA by these compounds is not due to nonspecific toxic effect or global RNA synthesis-inhibitory effect, but due to AR antagonistic effect. Thus, we obtained a new type of AR antagonists with non-steroidal/nonanilide-type structures. At present, we do not know why spectinabilin showed 100-fold potent inhibitory activity against DHT-induced PSA mRNA expression when compared with arabilin and SNF4435C. The precise mechanism for the inhibition of DHT-induced PSA mRNA expression by these compounds is now under investigation. In addition, the antitumor activities of these compounds in androgen-dependent and -independent prostate cancer cell functions will be reported elsewhere.

METHODS

General experimental procedures

Mass spectra were measured with JMS-T100LC mass spectrometer (JEOL, Tokyo, Japan). Optical rotations were made with P-1030 polarimeter (JASCO, Tokyo, Japan) using a micro-cell (light path 100 mm). UV spectra and IR spectra were recorded on U-1800 spectrophotometer (Hitachi High-Technologies, Tokyo, Japan) and FT-210 spectrometer (Horiba, Kyoto, Japan) in KBr disc, respectively. ^1H and ^{13}C NMR spectra were recorded on JNM-ECA600 spectrometer (JEOL) operating at 600 MHz and 150 MHz, respectively. A liquid chromatography (LC)-photo diode array (PDA)-MS system (Waters) with a photo diode array detector (2996) and mass analyzer (Micromass ZQ; Waters) was used for analysis and preparation.

Taxonomic studies

The producing strain, MK756-CF1, was isolated from a soil sample collected in Kochi prefecture, Japan. The morphological characteristics of strain

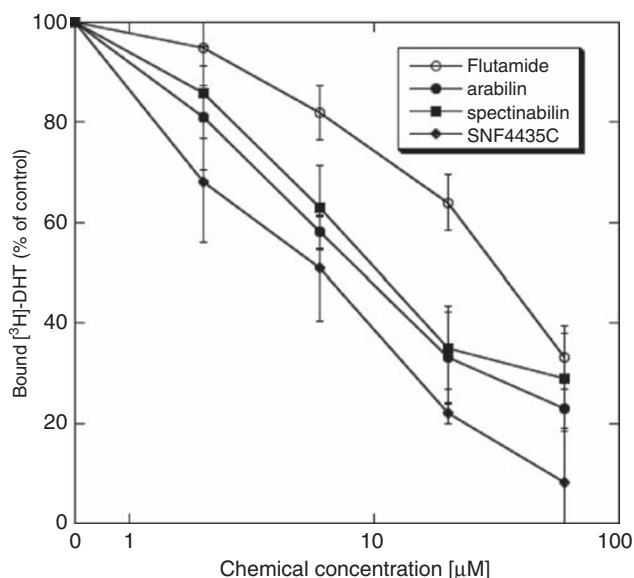


Figure 3 Effects of arabilin, spectinabilin, SNF4435C and flutamide on binding of dihydrotestosterone (DHT) to androgen receptor (AR). A 50 $\mu\text{g ml}^{-1}$ volume of maltose-binding protein-AR-ligand-binding domain (MBP-AR-LBD), 2 nM [^3H]DHT and the indicated concentrations of test compounds were incubated at 4°C for 3 h. Then, the radioactivity of [^3H]DHT bound to MBP-AR-LBD was measured with a liquid scintillation counter. Values are the means of four samples; bars, s.d.

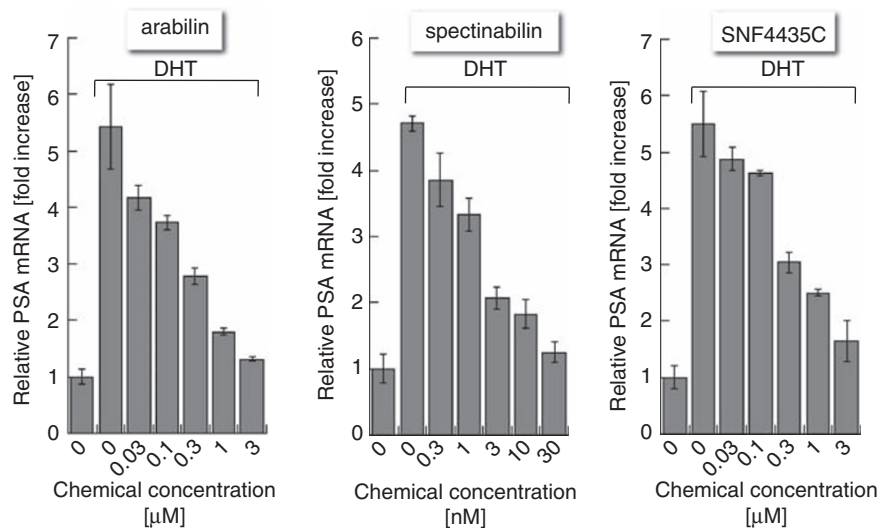


Figure 4 Effects of arabilin, spectinabilin and SNF4435C on dihydrotestosterone (DHT)-induced prostate-specific antigen (PSA) mRNA expression. LNCaP cells were treated with 0.1 nM of DHT and the indicated concentrations of test compounds. After 12 h, PSA mRNA were measured by real-time quantitative reverse transcription PCR. Values are the means of triplicate samples; bars, s.d.

MK756-CF1 were determined on yeast-starch agar. The 16S ribosomal RNA gene was amplified by PCR using genomic DNA of the strain and sequenced. The most related sequences were searched using the BLAST algorithm in the DNA Data Bank of Japan.

Fermentation

A slant culture of arabilin-producing organism was inoculated in a 500-ml baffled Erlenmeyer flask containing 110 ml of a seed medium consisting of galactose 2%, dextrin 2%, Bacto-soytone (Difco; BD, Franklin Lakes, NJ, USA) 1.0%, corn steep liquor (Oji Cornstarch, Tokyo, Japan) 0.5%, (NH₄)₂SO₄ 0.2% and CaCO₃ 0.2% in deionized water (pH 7.4 before sterilization). The culture was incubated on a rotary shaker (180 r.p.m.) at 27°C for 3 days. The seed culture (7 ml) of the strain was transferred into a 500-ml Erlenmeyer flask containing autoclaved press wheat (15 g) with deionized water (25 ml). The fermentation was carried out by a solid-state cultivation at 30°C for 14 days.

[³H] DHT-AR *in vitro* binding assay

This assay was performed according to the method described previously.¹⁵ In brief, the gene sequence corresponding to the ligand-binding domain (AR-LBD, 609–919 amino acids) in the C-terminus of AR was expressed in *Escherichia coli* strain DH5 α as a maltose-binding protein-fused protein (MBP-AR-LBD), followed by purification using amylose resin (Bio-Rad Laboratories, Hercules, CA, USA). Thus, the obtained recombinant MBP-AR-LBD (50 μ g ml⁻¹), [³H]DHT (2 nM) and test samples were incubated at 4°C for 3 h. Then, [³H]DHT-bound MBP-AR-LBD was precipitated with hydroxyapatite and radioactivity was measured with a liquid scintillation counter.

Detection of PSA mRNA by real-time reverse transcription PCR

Prostate cancer (LNCaP) cells were incubated in RPMI 1640 medium supplemented with 2% charcoal-stripped serum for 24 h. The cells were then treated with DHT (0.1 nM) and test compounds. After 12 h, RNA from the cells was isolated, and the expression of PSA genes was determined by real-time quantitative reverse transcription PCR, and normalized to GAPDH mRNA. The primer sequences used were as follows: for PSA, 5'-AGGTCGGAGTCAACGGATTT-3' (forward) and 5'-TAGTTGAGGTCAATGAAGGG-3' (reverse); for GAPDH, 5'-GGTCCTCACAGCTGCCATC-3' (forward) and 5'-CAGCCTGAGCGTAGCAGGT-3' (reverse).

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