Dissertation

Novel α-trifluoromethyl chalcone exerts antitumor effects against prostate cancer cells

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[Abstract]

Background: Despite treating advanced prostate cancer (PCa) with androgen deprivation therapy, it eventually progresses to castration-resistant PCa. Subsequently, taxanes are administered, but when PCa becomes resistant to taxanes, another treatment would be needed, which has not yet been established at present. We previously synthesized a novel α-trifluoromethyl chalcone, YS71, and reported its antitumor effects against PCa cells. In this study, we confirmed its efficacy against androgen-sensitive, androgen-independent, and taxane-resistant PCa cells.

Materials and Methods: In this study, the PCa cell lines used were LNCaP, PC-3, DU145, PC-3-TxR (paclitaxel-resistant), PC-3-TxR/CxR (paclitaxel- and cabazitaxel-resistant), DU145-TxR, and DU145-TxR/CxR. Their antiproliferative effects were evaluated using proliferation assay. The reverse transcriptase transcription–polymerase chain reaction and western blot were performed to determine the expression level of androgen receptor (AR), whereas luciferase assay was performed to determine the AR activity. Furthermore, TUNEL assay and western blot were performed to investigate the mechanism of the antiproliferative effect.

Results: YS71 exerted a dose-dependent antitumor effect on and inhibited AR activity and induced apoptosis in all PCa cells in a dose-dependent manner. Western blot showed that YS71 increased the apoptosis-related proteins, cleaved caspase-3 and cleaved PARP, and decreased the antiapoptotic proteins, Bcl-xL and Bcl-2. In addition, microarray analysis revealed that YS71 decreased several cancer-related genes.

Conclusion: YS71 exhibits antitumor activity by inducing apoptosis in PCa cells, including taxane-resistant cells. It is also a potential future therapeutic option for hormone- and chemotherapy-resistant PCa.

[Introduction]

Prostate cancer (PCa) is the most frequently diagnosed cancer among men and the second leading cause of cancer-related mortality in the United States (1). Most untreated PCas progress in an androgen-dependent manner; therefore androgen deprivation therapy (ADT) is effective for PCas (2). However, PCas gradually become resistant to ADT and eventually progress to castration-resistant prostate cancer (CRPC). Novel androgen receptor axis target (ARAT) agents, such as abiraterone, enzalutamide, apalutamide, and darolutamide, have been used to treat CRPC (3-6). However, even ARAT agents eventually fail to provide therapeutic benefits, and patients with CRPC who relapse following the administration of ARAT agents are subsequently treated with docetaxel (7). Furthermore, if CRPC becomes resistant to docetaxel, it is treated with cabazitaxel (8). Recently, the combination of docetaxel and hormone therapy has proven to be an effective initial treatment for metastatic PCa (CHAARTED trial) (9). At present, there is no clear treatment option for cabazitaxel-resistant CRPC. Thus, therapeutic agents for cabazitaxel-resistant CRPC need to be urgently developed.

Flavonoids are a class of polyphenolic compounds found in plant foods and beverages; they are known to exert diverse effects, including antiallergic, antiviral, anti-inflammatory, and vasodilatory effects (10). They have also been reported to exhibit antitumor activity against various malignant tumors, including PCa (11, 12). We have previously reported that 2'-hydroxyflavanone (2'-HF) inhibits AR activity against LNCaP, an androgen-sensitive PCa cell, and suppresses the growth of PC-3 and DU145, which are androgen-independent PCa cells (13). Furthermore, we prepared a derivative of 2'-HF, 16MS7F1924, and confirmed its potency against LNCaP, PC-3, and DU145 as

well as taxane-resistant PCa cells (14).

Chalcones are biosynthetic intermediates of flavonoids. They have attracted considerable attention owing to their wide range of biological activities, including anti-inflammatory, antioxidant, and even antitumor activities, like flavonoids, as well as their clinical potential (15, 16). We previously reported the synthesis of a novel α-trifluoromethyl chalcone obtained *via* Claisen–Schmidt condensation of an allyl methyl ketone with an aromatic aldehyde, demonstrating its enhanced antitumor effect, and designated it YS71 (17). In the present study, we compared the efficacy of YS71 in androgen-dependent, androgen-independent, and taxane-resistant PCa cells with that of the derivative of 2'-HF, 16MS7F1924.

[Material and Methods]

Derivatives and antibodies

The structural formula of YS71 is presented in Figure 1. YS71 was prepared according to the following reported method (17). Briefly, Claisen–Schmidt condensation of acetophenone with 3,4-difluorobenzaldehyde produced 3,4-difluoro chalcone. CF3 was inserted at the α-position using Togni reagent (18). The following antibodies were used for western blot: Mouse Anti-glyceraldehyde-3-phosphate Dehydrogenase (GAPDH; NB300-221) Antibody purchased from Novus Biologicals (Littleton, CO, USA); Rabbit Anti-histone H3 (D1H2, 4499S), Rabbit Anti-poly (ADP-Ribose) Polymerase (PARP; 46D11, 9532S), Rabbit Anti-cleaved PARP (5625s), Rabbit Anti-Bcl-xL (54H6, 2764s), Mouse anti-Bcl-2 (124, 15071s), and Horseradish Peroxidase (HRP)-Conjugated Anti-rabbit Immunoglobulin G (IgG; 7074) antibodies purchased

from Cell Signaling Technology (Danvers, MA, USA); Rabbit Anti-AR (ab133273) and Rabbit Anti-caspase-3 (ab32351) antibodies purchased from Abcam plc (Cambridge, UK); and HRP-Conjugated Anti-mouse IgG antibody purchased from Nacalai Tesque Inc. (Kyoto, Japan).

Cell culture

Human PCa cell lines LNCaP, PC-3, and DU145 were purchased from the American Type Culture Collection (Manassas, VA, USA). LNCaP (androgen-sensitive) and DU145 (androgen-independent) cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) containing1% penicillin/streptomycin (P/S; Thermo Fisher Scientific, Waltham, MA, USA) and 5% fetal bovine serum (FBS; Sigma-Aldrich) in a humidified incubator at 37° C with 5% CO2, respectively. PC-3 cells (androgen-independent) were cultured in Roswell Park Memorial Institute medium (RPMI-1640; Sigma-Aldrich) containing1% P/S and 5% FBS in a humidified incubator at 37° C with 5% CO2. PC-3-TxR, a paclitaxel-resistant cell line, PC-3-TxR/CxR, a paclitaxel- and cabazitaxel-resistant cell line, DU145-TxR, and DU145-TxR/CxR cell lines were developed in our laboratory as previously reported (19, 20). Previous studies have reported that PC-3-TxR and DU145-TxR cells are cross-resistant to docetaxel (21).

Proliferation assay

Cell proliferation assay was performed as follows: PC-3 and DU-145 were plated on six-well plates at a density of 2 × 104 cells. After 8 h, the cells were treated with 16MS7F1924 or YS71 for 72 h. The media were changed every 2 days, and reagents were added each time. LNCaP cells were seeded on six-well plates with DMEM-5%

FBS at a density of 5 × 104 cells. After 48 h, the media was replaced with DMEM-5% charcoal-stripped fetal calf serum (CCS; Cytiva, Tokyo, Japan), and the cells were treated with 16MS7F1924, YS71, and dihydrotestosterone (DHT) for 48 h. At the end of the culture period, the cells were harvested, and the number of cells was counted in triplicate using CellDrop BF (DeNovix, Wilmington, DE, USA) or a hemocytometer.

Reverse transcription–polymerase chain reaction

For the reverse transcription–polymerase chain reaction (RT-PCR), 48 h after seeding LNCaP cells on six-well plates with DMEM-5% FBS at a density of 3 × 105, the media was replaced with DMEM-5% CCS, and the cells were treated with YS71 for 24 h. In addition, total RNA was purified using Direct-zol RNA MiniPrep (Zymo Research, CA, USA). Complementary DNA (cDNA) was prepared via reverse transcription of total RNA (25 ng) using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). Each cDNA sample was amplified using Quick Taq HS DyeMix (TOYOBO, Japan). PCR reaction for GAPDH, AR, and prostate-specific antigen (PSA) was performed as previously described (22). Each of the amplified PCR product was identified via electrophoresis on a 1.5% agarose gel.

Luciferase assay

Luciferase assay was essentially performed according to our previous study (12). About 24 h after transfection, LNCaP and PC-3 cells were treated with 16MS7F1924, YS71, and DHT for 24 h. After the treated cells were harvested, the cells were lysed with luciferase lysis buffer (Promega, Madison, WI, USA), and luciferase activity was quantified using a luminometer as previously described (13).

Western blot analysis

For the western blot analysis of the AR protein, 48 h after seeding LNCaP cells on six-well plates with DMEM-5% CCS at a density of 3×10^5, the cells were treated with the indicated concentration of YS71 for 20 h (for whole-cell proteins) or 8 h (for nuclear proteins) in the presence or absence of 10-nM DHT. For the western blot analysis of the apoptotic protein, 24 h (LNCaP) or 8 h (PC-3 and DU145 cells) after seeding PCa cells on six-well plates with DMEM-5% FBS (LNCaP and DU145 cells) or RPMI-5% FBS (PC-3 cells) at a density of 5×10^5, PCa cells were treated with YS71 for 24 h. For whole-cell protein extraction, cell lysates were prepared using the M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific) containing 1% protease inhibitor cocktail (Sigma-Aldrich). For nuclear protein extraction, cell lysates were prepared using a cytoplasmic and nuclear protein extraction kit (101Bio) according to the manufacturer's protocols. Each sample was mixed with lithium dodecyl sulfate sample buffer and a sample reducing agent (Thermo Fisher Scientific) and proteins were separated *via* dodecyl sulfate–polyacrylamide gel electrophoresis. The separated proteins were transferred to nitrocellulose membranes. To prevent binding of nonspecific antibodies to the membrane surface, the membranes were blocked with 1% gelatin in 0.05% Tween in Tris-buffered saline (TBS-T) for 1 h at room temperature. The membranes were shaken overnight at 4°C with primary antibodies according to the manufacturer's protocols. The membranes were washed with TBS-T 3 times and shaken with HRP-Conjugated Anti-rabbit or Anti-mouse secondary antibodies for 1 h, then washed 4 times with TBS-T. Protein bands were detected using Clarity Max Western ECL Substrate (Bio-Rad Laboratories) and analyzed using Image Lab v6.1.0 (Bio-Rad Laboratories).

Apoptosis assay

To determine whether YS71 induces apoptosis in PCa cells, DeadEnd Fluorometric rTdT-Mediated dUTP Nick-End Labeling (TUNEL) Assay Kit (Promega) was used according to the manufacturer's protocols. Briefly, 3×10^4 PCa cells were seeded overnight on presterilized slide coverslips on eight-well plates and exposed to 0–3-μg/mL YS71 for 8 h. The cells were fixed in 4% methanol-free formaldehyde solution in phosphate-buffered saline (PBS) for 25 min, washed with PBS twice, and permeabilized with 0.2% Triton X-100 solution in PBS for 5 min. After rinsing twice with PBS, excess liquid was removed and each slide was covered with equilibration buffer for 10 min. The equilibration buffer was blotted off and rTdT incubation buffer was added to each slide and incubated at 37°C for 1 h in the dark. The reactions were terminated by immersing the slides in sodium chloride sodium citrate for 15 min. The slides were washed three times with PBS, and chromosomal DNA was stained with 4′,6-diamidino-2-phenylindole (Sigma-Aldrich) for 15 min. After washing 3 times with PBS, the stained cells mounted on the slides were examined using OLYMPUS cellSens Standard (OLYMPUS, Tokyo, Japan). The final images were processed using Adobe Photoshop CC (Adobe, San Jose, CA, USA).

Microarray analysis

Microarray analysis was conducted to investigate the genetic changes induced by YS71 treatment. PC-3 and DU145 cells were treated with YS71 for 0 and 24 h, respectively, after being seeded on six-well plates at a density of 15×10^4. Total RNA was purified using RNeasy Mini Kit (QIAGEN, Venlo, the Netherlands) and sent to Takara Bio (Shiga, Japan).

Statistical analysis

Statistical analyses were conducted using the commercially available software GraphPad Prism (GraphPad Software, San Diego, CA, USA). Student's *t*-test was employed to evaluate between-group differences. Significance was defined as $*P < .05$, $**P < .01$, $***P < .001$, and $****P < .0001$.

[Result]

YS71 inhibited proliferation in PCa cells

To investigate the antiproliferative activity of YS71 against PCa cells, androgen-sensitive LNCaP cells and androgen-independent PC-3 and DU145 cells were treated with 16MS7F1924 and YS71. As previously reported, 16MS7F1924 inhibited cell proliferation in LNCaP cells in a dose-dependent manner in the presence or absence of DHT (IC_{50}: 1.34 and 1.15 μM, respectively). YS71 demonstrated a significantly stronger inhibition of cell proliferation than 16MS7F1924 in LNCaP cells in the presence or absence of DHT (IC_{50}: 0.59 and 0.7 μM, respectively) (Figure 2A). Furthermore, YS71 demonstrated a significantly stronger inhibition of cell proliferation than 16MS7F1924 in androgen-independent PC-3 cells (IC_{50}: 0.54 and 0.35 μM, respectively) and DU145 cells (IC_{50}: 0.45 and 0.26 μM, respectively) (Figure 2B and 2C).

YS71 inhibits AR activity of LNCaP and PC-3 as well as AR-V7 activity of PC-3

Because YS71 suppressed DHT-induced cell proliferation in LNCaP cells, we next investigated its effect on DHT-induced PSA expression. RT-PCR showed that YS71 reduced the messenger RNA (mRNA) levels of PSA induced by 10-nM DHT (Figure

3A). To investigate the mechanism of PSA suppression, we also transfected LNCaP cells with a luciferase reporter (pGL3PSAp-5.8) driven by the PSA promoter regulated by the AR; moreover, we studied the PSA promoter activity *via* luciferase assay. YS71 was found to suppress the PSA promoter activity induced by DHT in a dose-dependent manner (Figure 3B). To further investigate, we explored the changes in AR expression levels in LNCaP cells. RT-PCR revealed that the mRNA expression level of AR was reduced by YS71. Furthermore, western blot analysis showed that YS71 suppressed the AR protein expression levels in LNCaP cells and the DHT-induced nuclear translocation of AR (Figure 3C). These results indicate that transcriptional or post-transcriptional regulation by YS71 may play a role in AR expression.

We also examined the effect of YS71 on the activity of wild-type AR and AR-V7, one of the splicing variants of AR with a strong androgen-independent activity. Promoter activity was investigated for PC-3 cells transfected with pGL3PSAp-5.8 and pEGFP-AR or pEGFP-AR-V7. In both wild-type AR and AR-V7, the PSA promoter activity was suppressed by YS71 at a significantly lower concentration than 16MS7F1924 (Figure 4).

YS71 inhibits proliferation of taxane-resistant PCa cells

To investigate the inhibitory effect of YS71 on the proliferation of docetaxel-resistant and cabazitaxel-resistant strains in PC-3 and DU145, respectively, we conducted proliferation assays by adding 16MS7F1924 and YS71 (Figure 5). YS71 significantly inhibited the proliferation of all these PCa cells in a dose-dependent manner at a lower concentration than 16MS7F1924 (16MS7F1924: the IC_{50} of PC-3-TxR, PC-3-TxR/CxR, DU145-TxR, and DU145-TxR/CxR cells were 0.59, 0.48, 0.45, and 0.52 μM, respectively. YS71: the IC_{50} of PC-3-TxR, PC-3-TxR/CxR, DU145-TxR, and DU145-

TxR/CxR cells were 0.35, 0.18, 0.18, and 0.20 μM, respectively)

YS71 inhibits tumor growth by inducing apoptosis

To investigate the mechanism of the antitumor effect of YS71 on PCa cells, we conducted TUNEL assay. YS71 induced apoptosis in LNCaP, DU145, DU145-TxR, DU145-TxR/CxR, PC-3, PC-3-TxR, and PC-3-TxR/CxR cells in a dose-dependent manner (Figure 6). Apoptosis-related and antiapoptotic proteins were also analyzed *via* western blot analysis. Treatment of PCa cells with YS71 resulted in a dose-dependent increase in apoptosis-related proteins, cleaved caspase-3 and cleaved PARP, and a dose-dependent decrease in antiapoptotic proteins, Bcl-xL and Bcl-2 (Figure 7). Note that Bcl-2 is rarely expressed in the DU145 family.

YS71 treatment decreased several cancer-related genes

To further elucidate the antitumor effect of YS71, we examined the genetic changes after treatment with YS71. The numbers of genes with expression levels reduced to 1/3 or less by the treatment of YS71 were 2,743 for PC-3 and 2,990 for DU145, of which 340 genes were commonly reduced in both cases (Figure 8). Table 1 presents some of the commonly reduced genes that were particularly affected (approximately 1/10–1000). Many of these genes have been previously reported to be associated with cancer cell proliferation, and some were also found to be associated with PCa. YS71 reduced these genes, suggesting an association between YS71 and antitumor effects (23-28).

［**Discussion**］

Fluorine has the unique properties of small atomic size and high electronegativity. It influences the acid dissociation constant, intrinsic potency, molecular structure, binding affinity to target molecules, membrane permeability, metabolic pathways, and pharmacokinetic properties of bioactive molecules (29). We previously focused on this property and determined whether the insertion of fluorine or fluorinated functional groups into synthetic chalcones could enhance their activity and successfully synthesize YS71 (17). YS71 suppressed cell proliferation of PC-3, DU145, and their paclitaxel- and cabazitaxel-resistant strains; moreover, it exhibited antitumor activity against PC-3 xenografts in severe combined immunodeficient mice. 16MS7F1924, which was selected as the control group in this study, is a chalcone derivative known to exert potent antitumor effects on PCa (14). 16MS7F1924 induced apoptosis *via* the Akt pathway in a dose-dependent manner and safely suppressed tumor growth in all cell lines, including taxane-resistant PCa cells *in vivo*. YS71, a derivative synthesized based on the results with 16MS7F1924, exerted more potent antitumor effects than 16MS7F1924 on all PCa cells.

Although the mechanism of the antitumor effect of YS71 is still unknown, we propose the following hypothesis as to why YS71 exerts stronger antitumor effects than 16MS7F1924. One of the major differences between the two compounds is whether the linker double bond connecting the two aromatic rings is cis or trans, which significantly changes the overall steric structure of the molecule. Aromatic rings are considered to be among the most important pharmacophores owing to their planarity, π-interactions with biomolecules, and other important functions. Furthermore, the α,β-unsaturated ketones of both can act as Michael acceptors and react with nucleophilic molecules in biomolecules to alkylate them. Fluorine is a very strong electron-

withdrawing group, and it is possible that YS71 with CF3 attached at the α-position acts more strongly as a Michael acceptor, which may influence its action. The proof of these hypotheses will be the subject of future research.

PCa is usually heterogeneous, and simultaneous treatment of hormone-sensitive and hormone-independent PCa cells may play a significant role in PCa therapy, as indicated by the results of the CHAARTED Trial (9). One of the factors in the pathogenesis of CRPC is the activation of AR signaling pathway (30). Such an activation can be caused by a variety of factors, including AR splicing variants, AR mutations, AR amplification, and ligand-independent activation. In addition, AR signaling pathway activation is known to suppress apoptosis and increase cell proliferation; it has also been implicated in the pathogenesis of CRPC (31, 32). Our results indicate that YS71 suppresses AR activity in LNCaP by downregulating AR expression and suppressing nuclear translocation. YS71 also suppressed the activity of not only wild-type AR but also AR-V7 transfected into PC-3 cells. In this study, YS71 suppressed AR mRNA expression in LNCaP, but whether this was due to suppressed AR transcription or reduced AR stability is unknown and is an agenda topic for future studies.

Microarrays revealed that several cancer-related genes were repressed by YS71 in PCa cells. Currently, we are investigating the relationship between some of the genes and the mechanism of action of YS71. For example, pre-B-cell leukemia homeobox-1 (PBX1) is a member of the three-amino-acid-loop-extension class of homeodomains and is known to regulate the expression of genes involved in human development (33). In a variety of cancers, including breast, lung, and gastric cancers, PBX1 is dysregulated, affecting proliferation, metastasis, and chemotherapy resistance (34-36). As an oncogenic transcription factor, PBX1 is reportedly highly

expressed in most PCa tissues, promoting PCa cell proliferation and chemotherapy resistance to several anticancer drugs (24). Activated leukocyte cell adhesion molecule (ALCAM) is a transmembrane protein of the immunoglobulin superfamily (Ig-SF) (37, 38). It has been identified as a substrate of a disintegrin and metalloprotease (ADAM) 17, and its activity is promoted by epidermal growth factor and transforming growth factor-β (TGF-β) (39-41). Furthermore, it has been reported to be associated with the progression of several cancers, including breast cancer, gastric cancer, and melanoma (38). ALCAM is also known to play an important role in the bone metastasis of PCa, and elevated ALCAM exhibits prognostic ability comparable to that of PSA for PCa metastasis and survival (25, 41). Bone morphogenetic protein 7 (BMP7) is a member of the TGF-β family of proteins secreted by bone stem cells and is thought to contribute to the suppression of cancer cell migration and invasion by inhibiting epithelial–mesenchymal transition (EMT) (42). Several studies have reported that BMP7 expression in PCa cell lines is inversely correlated with tumorigenic and metastatic potentials (23, 43, 44). The suppression of BMP7 by YS71 appears to be a contradictory result to the antitumor effects of YS71. However, because BMP7 is known to promote bone remodeling, it may be associated with bone metastasis in PCa, which tends to adopt an osteogenic phenotype (45). Although the relationship between these genes and YS71 is still unclear, elucidation of the mechanism of action of YS71 is expected to further improve its pharmacological effects.

These results indicate that YS71 has a broad antitumor effect on androgen-sensitive PCa cells as well as taxane-resistant PCa cells and may be a new therapeutic option for CRPC.

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[Figures and Tables]

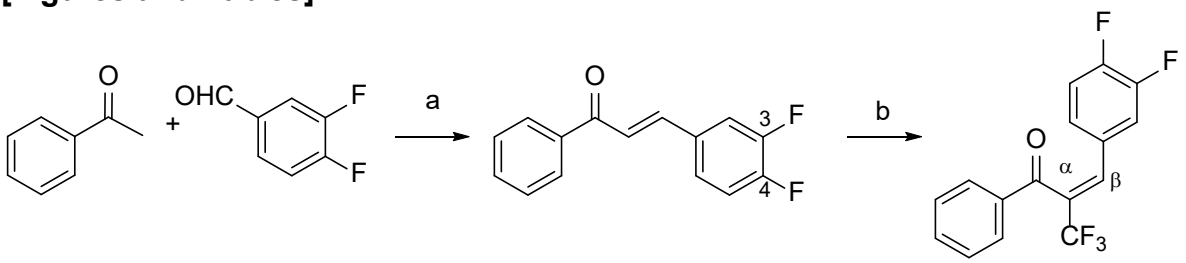

Figure 1. Chemical structure of YS71 and antiproliferative effect of 16MS7F1924.

YS71 was obtained *via* synthesis of 3,4-difluoro-α-trifluoromethyl chalcone (αTFMC). Reagents and conditions: (a) 40% KOH, EtOH, room temperature, (b) 1-trifluoromethyl-1,2-benziodoxol-3(1H)-one (Togni reagent), CuI, DMF, 80°C.

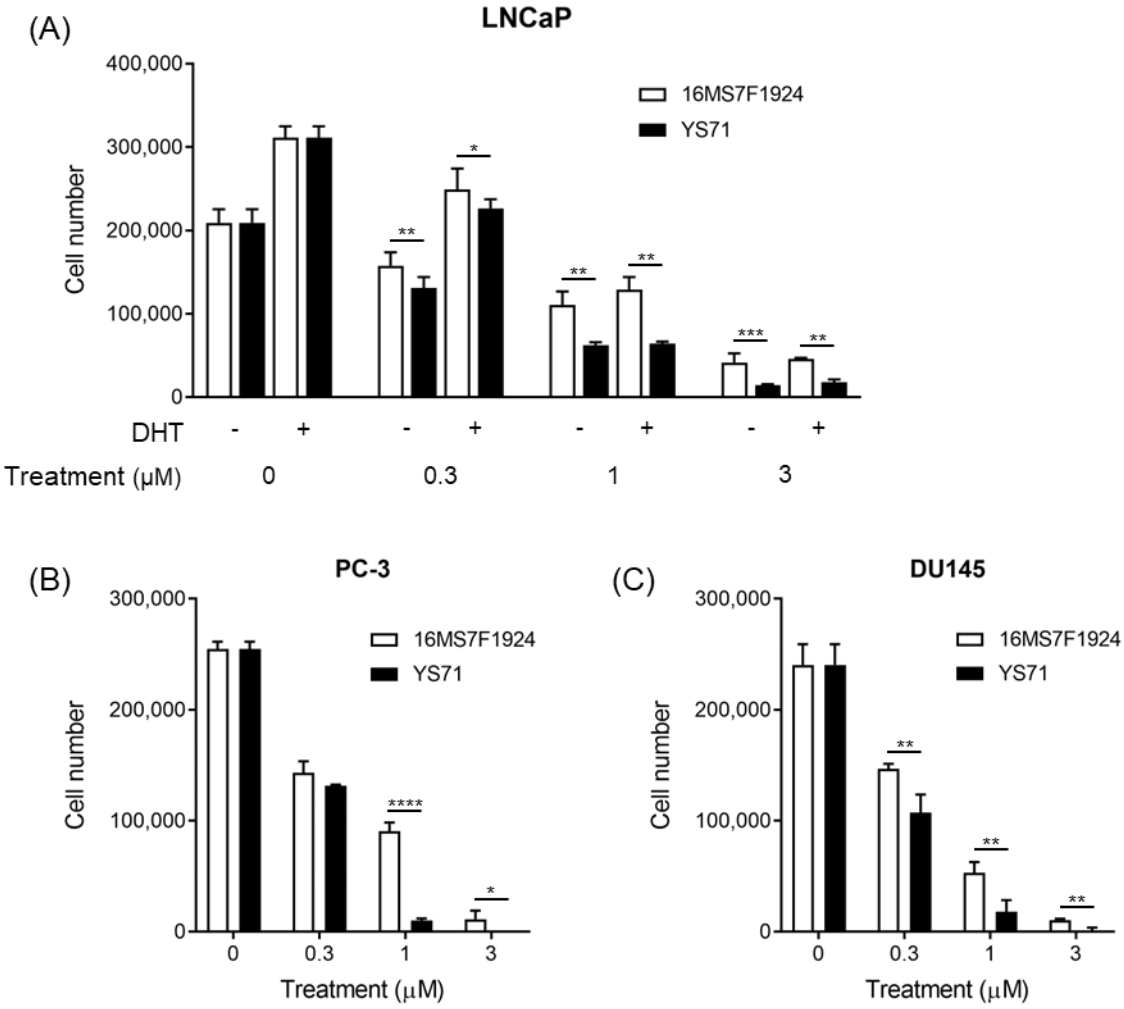

Figure 2. Antiproliferative effect of YS71 on PCa cells. (A) Proliferation assay of LNCaP. LNCaP cells were treated with 16MS7F1924 or YS71 for 72 h in the presence or absence of DHT. (B and C) Proliferation assay of PC-3 and DU145. PC-3 and DU145 cells were treated with 16MS7F1924 or YS71 for 72 h. Data are expressed as mean ± standard deviation (SD). DHT, dihydrotestosterone

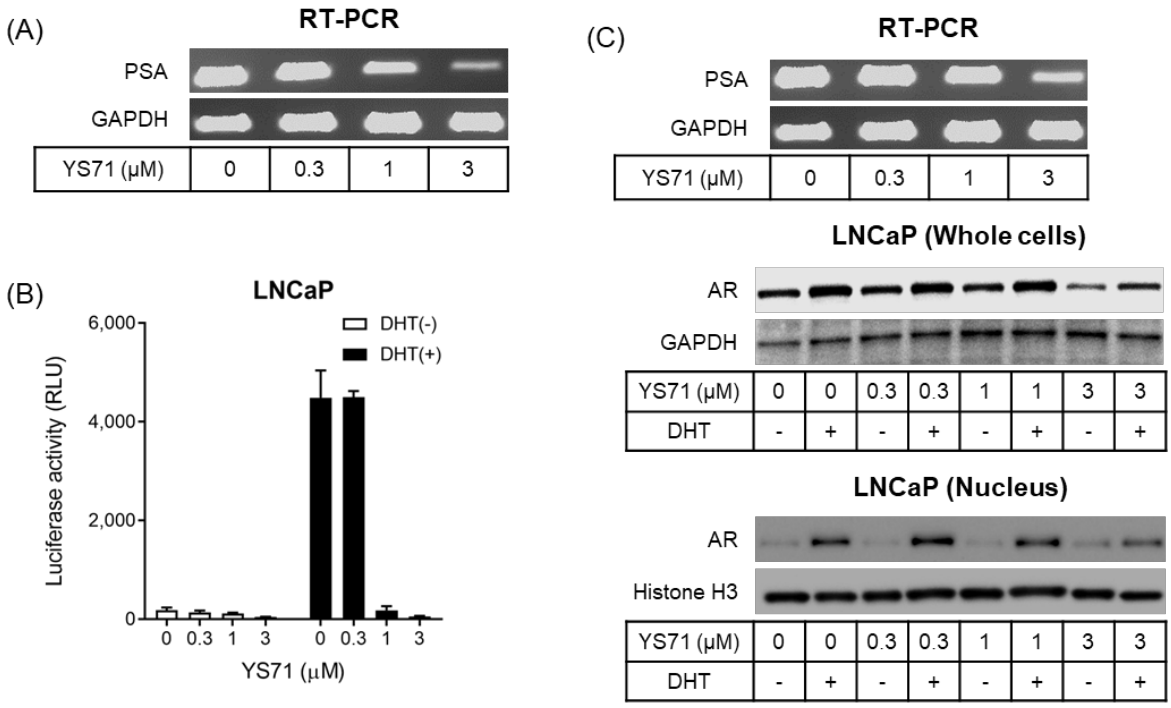

Figure 3. Effect of YS71 on AR activity in LNCaP. (A) RT-PCR was performed to confirm the mRNA expression of PSA in LNCaP cells treated with YS71 and DHT for 24 h. (B) Luciferase assay of PSA promoter in LNCaP cells. About 24 h after transfection of LNCaP cells with a luciferase reporter plasmid (pGL3PSAp-5.8), LNCaP cells were treated with YS71 for 24 h in the presence or absence of 10-nM DHT, and luciferase activity was measured. (C) RT-PCR was performed to confirm the mRNA expression of AR in LNCaP cells treated with YS71 and DHT for 24 h. Western blot analysis was conducted to confirm that DHT-induced AR protein expression and AR nuclear translocation were regulated by YS71 in LNCaP cells treated with YS71 for 20 h (for total cellular proteins) and 8 h (for nuclear proteins) in the presence or absence of 10-nM DHT. Histone H3 was used as a loading control for nuclear proteins. Data are expressed as mean ± standard deviation (SD). PSA, prostate-specific antigen; AR, androgen receptor; DHT, dihydrotestosterone

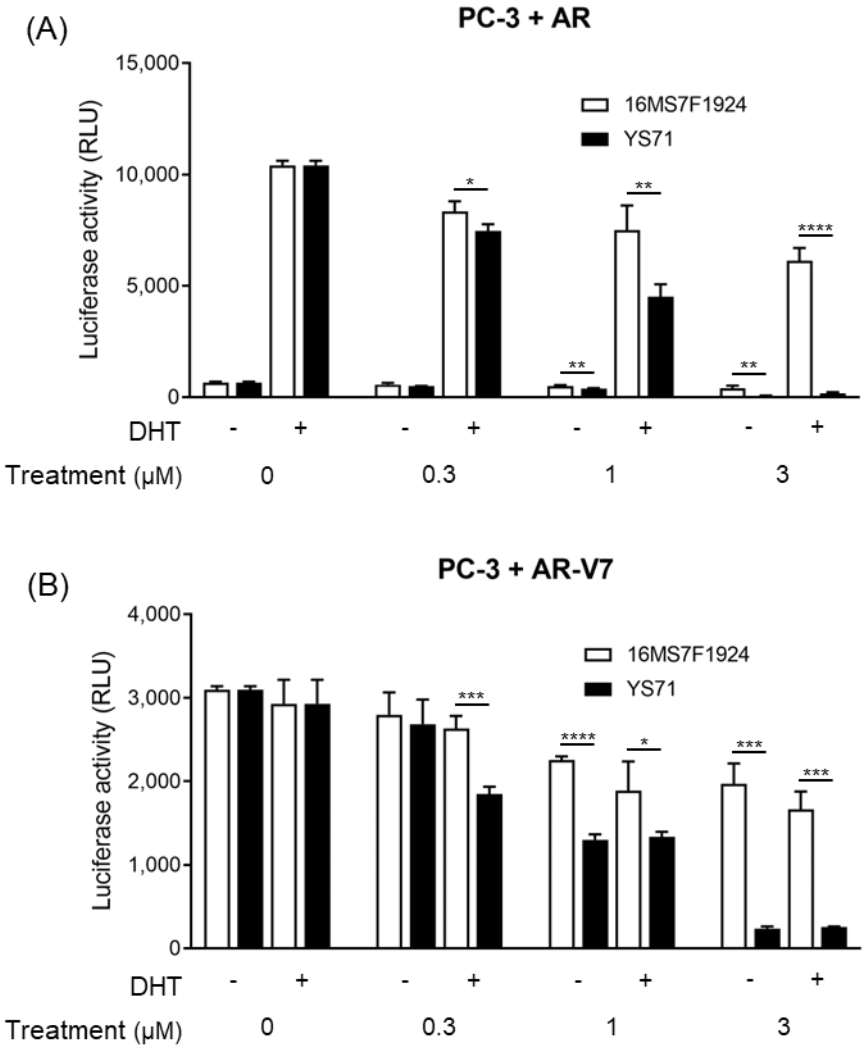

Figure 4. Effect of YS71 on AR activity in PC-3 transfected with wild-type AR and AR-V7. PC-3 cells were transfected with the luciferase reporter plasmid pGL3PSAp-5.8 with (A) pEGFP-AR plasmid or (B) pEGFP-AR-V7 plasmid for 24 h and then treated with the indicated concentrations of 16MS7F1924 or YS71 in the presence or absence of 10-nM DHT. After 24 h, luciferase assay was performed. Data are expressed as mean ± standard deviation (SD). AR, androgen receptor; DHT, dihydrotestosterone

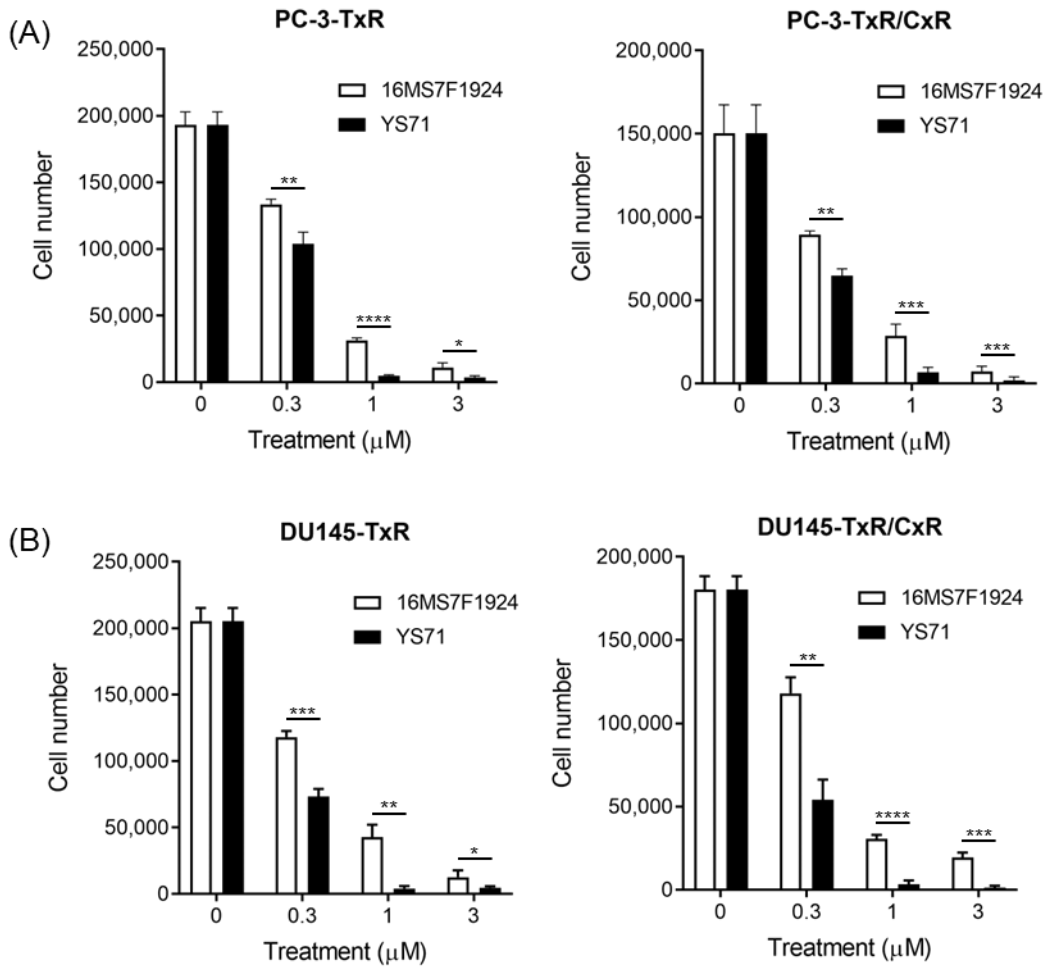

Figure 5. Antiproliferative effect of YS71 on taxane-resistant PCa cells. (A and B), PC-3-TxR (paclitaxel-resistant), PC-3-TxR/CxR (paclitaxel- and cabazitaxel-resistant), DU145-TxR, and DU145-TxR/CxR cells were treated with 16MS7F1924 or YS71 for 72 h. Data are expressed as mean ± standard deviation (SD).

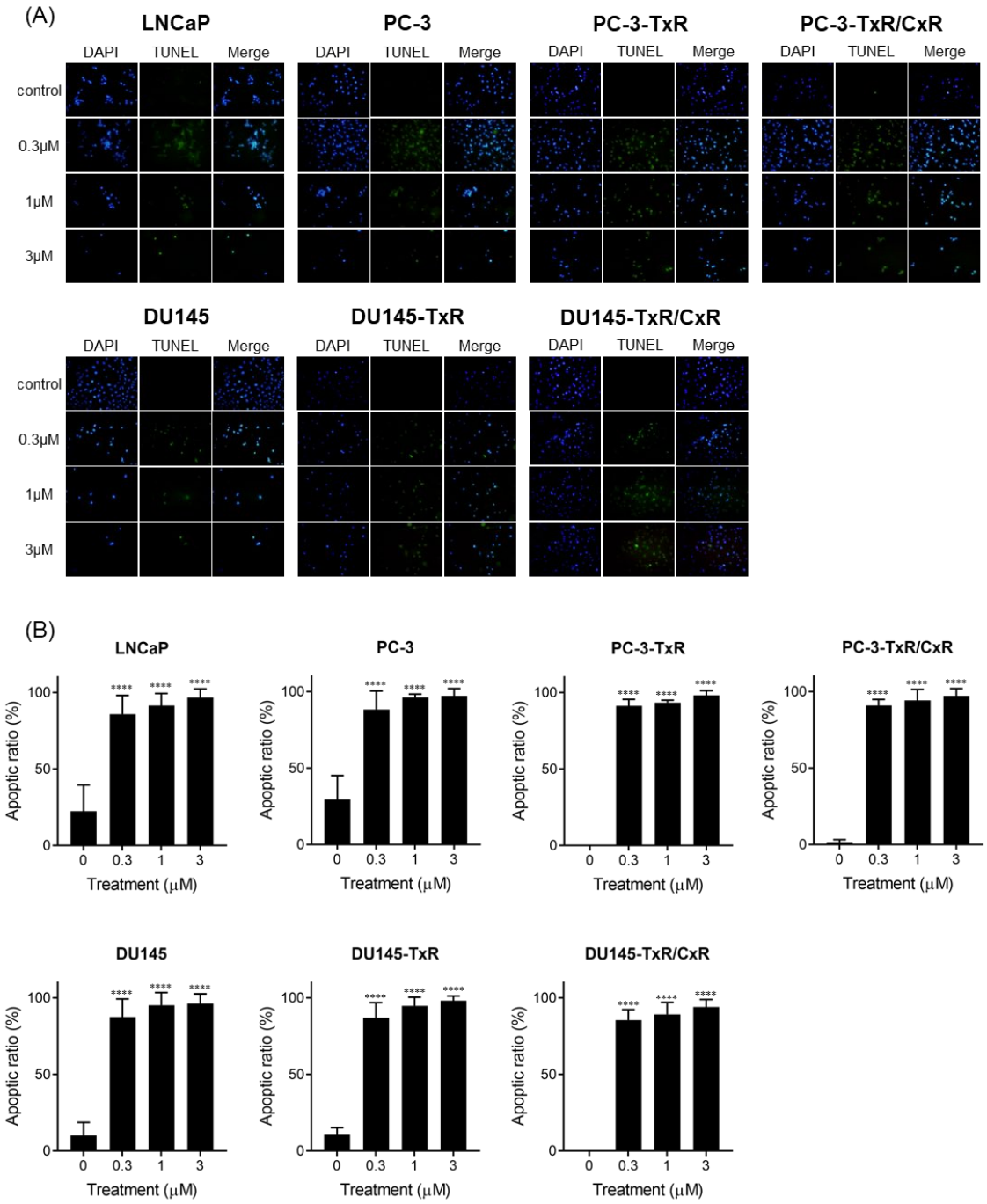

Figure 6. Apoptosis assay of PCa cells treated with YS71. (A) TUNEL assay of PCa cells. PCa cells were treated with the indicated concentration of YS71 for 8 h, and TUNEL assay was conducted. (B) The apoptosis ratio was calculated. Data are expressed as mean ± standard deviation (SD). TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling

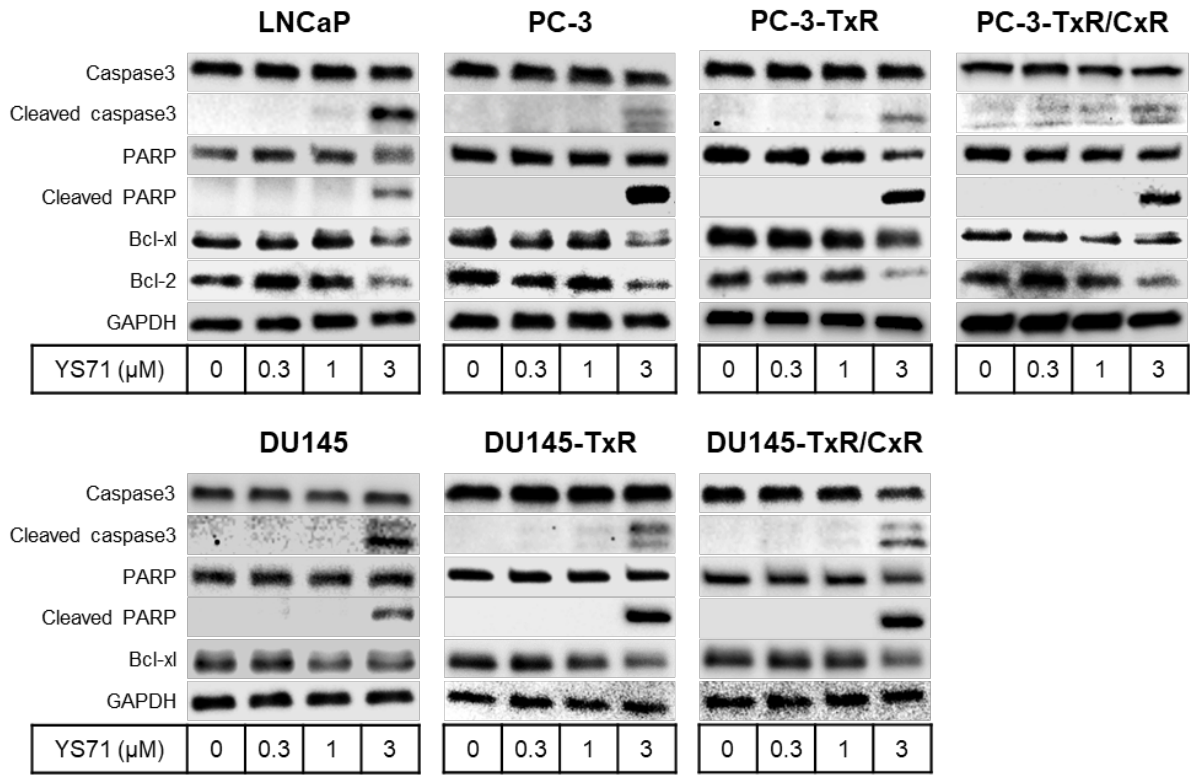

Figure 7. Western blot analysis of PCa cells. PCa cells were treated with the indicated concentrations of YS71 for 48 h. Western blot analysis was conducted for apoptotic proteins caspase-3 (32 kD), cleaved caspase-3 (17 kD), PARP (116 kD), and cleaved PARP (89 kD) and antiapoptotic proteins Bcl-xL (30 kD) and Bcl-2 (26 kD).

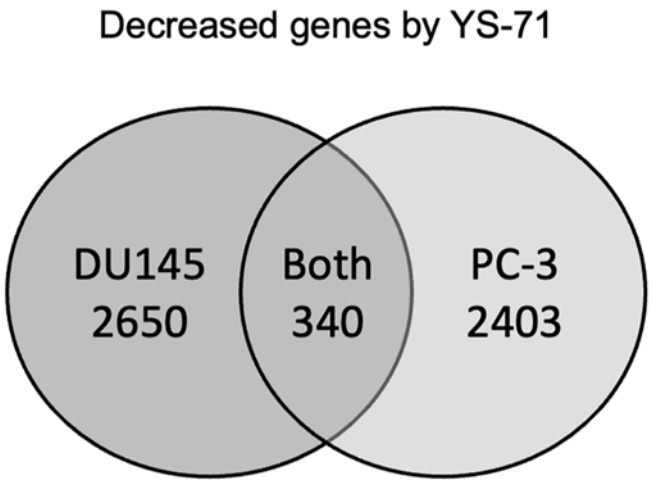

Figure 8. Microarray analysis of PCa cells. The number of genes with expression reduced to 1/3 or less by the treatment of YS71 is presented. It shows that expression reduced in PC-3 only, DU145 only, and in common, respectively.

Name	Genbank accession	Ratio of expression 24 h after treatment	
		DU145	PC-3
Bone morphogenetic protein 7 (BMP7)*	NM_001719	0.0016	0.0054
PBX homeobox 1 (PBX1)*	NM_001353130	0.0020	0.0022
Activated leukocyte cell adhesion molecule (ALCAM)*	NM_001627	0.0026	0.2161
Secretory leukocyte peptidase inhibitor (SLPI)*	NM_003064	0.0920	0.0063
Contactin 1 (CNTN1)*	NM_001843	0.0117	0.1096
S100 calcium binding protein A4 (S100A4)*	NM_002961	0.0371	0.1201
Calbindin 1 (CALB1)	NM_004929	0.1568	0.0004
Beta-secretase 2 (BACE2)	NM_012105	0.0013	0.0005
Claudin 4 (CLDN4)	NM_001305	0.1725	0.0009
KH RNA binding domain containing, signal transduction associated 3 (KHDRBS3)	NM_006558	0.0340	0.0014
Fibroblast growth factor binding protein 1 (FGFBP1)	NM_005130	0.0923	0.0020
Ninein like (NINL)	NM_025176	0.0051	0.0025
Growth factor receptor bound protein 14 (GRB14)	NM_004490	0.0027	0.0455
ATP binding cassette subfamily A member 13 (ABCA13)	NM_152701	0.0227	0.0029
Long intergenic non-protein coding RNA 2582 (LINC02582)		0.0693	0.0038
EPH receptor A4 (EPHA4)	NM_004438	0.0060	0.0211
Prickle planar cell polarity protein 1 (PRICKLE1)	NM_153026	0.0069	0.0094
G protein-coupled estrogen receptor 1 (GPER1)	NM_001039966	0.1106	0.0076
Integrin subunit beta 4 (ITGB4)	NM_000213	0.2496	0.0079
Vesicle associated membrane protein 8 (VAMP8)	NM_003761	0.2695	0.0108

*Association with prostate cancer has been reported.

Table I. Partial list of genes reduced by YS71 administration.