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ORIGINAL ARTICLE

Pharmacological inhibition of androgen receptor expression induces cell death in prostate cancer cells

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Abstract

The androgen receptor (AR) plays an important role in the pathogenesis and development of prostate cancer (PCa). Mostly, PCa progresses to androgen-independent PCa, which has activated AR signaling from androgen-dependent PCa. Thus, inhibition of AR signaling may be an important therapeutic target in androgen-dependent and castration-resistant PCa. In this study, we determined the anticancer effect of a newly found natural compound, sakurasosaponin (S-saponin), using androgen-dependent and castration-resistant PCa cell lines. S-saponin induces mitochondrial-mediated cell death in both androgen-dependent (LNCaP) and castration-resistant (22Rv1 and C4-2) PCa cells, via AR expression. S-saponin treatment induces a decrease in AR expression in a time- and dose-dependent manner and a potent decrease in the expression of its target genes, including prostate-specific antigen (PSA), transmembrane protease, serin 2 (TMPRSS2), and NK3 homeobox 1 (NKX3.1). Furthermore, S-saponin treatment decreases B-cell lymphoma-extra large (Bcl-xL) and mitochondrial membrane potential, thereby increasing the release of cytochrome c into the cytosol. Moreover, Bcl-xL inhibition and subsequent mitochondria-mediated cell death caused by S-saponin were reversed by Bcl-xL or AR overexpression. Interestingly, S-saponin-mediated cell death was significantly reduced by a reactive oxygen species (ROS) scavenger, *N*-acetylcystein. Animal xenograft experiments showed that S-saponin treatment significantly reduced tumor growth of AR-positive 22Rv1 xenografts but not AR-negative PC-3 xenografts. Taken together, for the first time, our results revealed that S-saponin induces mitochondrial-mediated cell death in androgen-dependent and castration-resistant cells through regulation of AR mechanisms, including downregulation of Bcl-xL expression and induction of ROS stress by decreasing mitochondrial membrane potential.

Keywords Prostate cancer · Sakurasosaponin · Androgen receptor · Bcl-xL

Introduction

Prostate cancer (PCa) is one of the most common cancers in men in the USA, and its mortality rate is high [1, 2]. PCa is dependent on androgen, and androgen activates the transcriptional activity of the androgen receptor (AR) by

In-Sung Song and Yu Jeong Jeong contributed equally to this work.

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binding with the AR and mediating the growth of PCa cells [3–5]. For patients, hormone therapy [androgen deprivation therapy (ADT)] inhibits ligands from binding to receptors or inhibits the activity of AR by androgen blockade [6]. Despite ADT, however, most patients progress to castration-resistant PCa (CRPC) without adequate chemotherapy [7]. It is hypothesized that the amplification of AR and the mutation of AR results in the development of CRPC, which means that the AR is still functional and activates target genes of AR in CRPC [4, 8, 9]. Therefore, it has been suggested that the downregulation of AR expression should be considered as a key strategy for the treatment of all stages of PCa. Currently, three classes of agents are used for AR signaling targeting. These classes are used to block the activity of the AR, inhibit endogenous androgen production, and decrease the stability of AR protein [10–13].

The anti-apoptotic protein B-cell lymphoma-extra large (Bcl-xL) interacts with pro-apoptotic member Bax or Bim to protect the cell from death by controlling mitochondrial membrane potential and volume and consequently blocks the release of cytochrome c into the cytoplasm [14, 15]. Bcl-xL also may prevent apoptosis through a cytochrome c-independent pathway [16, 17]. The Bcl-xL protein is usually regulated at the gene expression level, but posttranscriptional regulation is also possible [18]. Bcl-xL has been reported to play an important role in the progression of PCa and to be highly expressed in metastatic PCa and CRPC [19–21]. In addition, AR has been reported to interact with the bcl-x promoter, and abolishing the AR gene in PCa cells significantly inhibited Bcl-xL expression [22, 23]. Thus, modulating AR expression may be an important strategy to inhibit PCa.

In this study, we screened an in-house plant-derived single compound library to identify compounds that induced apoptosis in PCa cells. We identified sakurasosaponin (S-saponin) as a novel AR inhibitor from the screening hits and defined its mechanism of action in PCa cells. S-saponin, a newly identified saponin from the leaves of *Aegiceras corniculatum* and roots of *Jacquinia flammea* Millsp, has been reported to inhibit the growth of various cancers such as breast cancer, lung cancer, colon cancer, and melanoma [24–26]. However, the effects of S-saponin on PCa have not yet been elucidated.

Here, we demonstrated that S-saponin significantly induced mitochondrial-mediated cell death in AR-expressing PCa cells without affecting the mitochondrial-dependent mechanism in AR-negative PCa cells. We also confirmed that the pro-survival protein Bcl-xL, an AR target gene, was inhibited by treatment with S-saponin, which is important in S-saponin-induced mitochondria-mediated cell death. The xenograft and orthoxenograft experiments indicated that S-saponin treatment largely decreased tumor volumes in vivo. These results suggest that S-saponin might be a novel therapeutic agent for AR-expressing androgen-dependent and castration-resistant PCa patients.

Materials and methods

Cell culture, antibodies, and chemicals

The PCa cell lines LNCaP, 22Rv1, PC-3, and DU145 were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA), while the C4-2 cells were a kind gift from Dr. Sang-Jin Lee (Genitourinary Cancer Branch, Center for Prostate Cancer, Research Institute, Hospital of National Cancer Center, Republic of Korea). These cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) at 37 °C, in a humidified incubator containing 5% CO_2. Antibodies against poly-(adenosine diphosphate-ribose) polymerase (PARP), Bcl-xL, and caspase-3 were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies against Prx3 and hemagglutinin epitope (HA) were purchased from AbFrontier (Seoul, Korea). Antibodies against AR, TMPRSS2, NKx3.1, and tubulin were purchased from Santa Cruz Biotechnology (CA, USA). Antibodies to cytochrome C were purchased from BD Pharmingen (San Jose, CA, USA). Flag antibody and *N*-acetyl-L-cysteine (NAC) were purchased from Sigma (St. Louis, MO, USA). All the chemicals not mentioned above were obtained from Sigma (St. Louis, MO, USA).

Extraction and isolation of sakurasosaponin

Primula sieboldii were purchased from Somang-Nongwon farm, Daejeon, Korea, and identified by Professor Dae-Keun Kim, Woosuk University, Jeonju, Korea. The specimen is reserved in the Laboratory of Natural Products Chemistry, Kyunghee University, Suwon, Korea.

The air-dried roots of *P. sieboldii* (200 g) were extracted with 80% MeOH (3 L × 3), and the concentrated extract (32.4 g) was poured in H_2O (500 mL) and extracted with EtOAc (500 mL × 3) and *n*-BuOH (500 mL × 3), successively. Each layer which was concentrated in vacuo gave the EtOAc fraction (PSE, 4.7 g), *n*-BuOH fraction (PSB, 12.8 g), and aqueous fraction (PSW, 14.9 g), respectively. The PSB was subjected to a silica gel column (8 × 15 cm) chromatography and eluted with $CHCl_3$-MeOH (10:1 → 5:1 → 3:1, 2L of each) and $CHCl_3$-MeOH-H_2O (10:3:1 → 7:3:1 → 65:35:10, 2L of each) to produce 12 fractions (PSB1 to PSB12). Fraction PSB9 (8.4 g) was applied to octadecyl silica gel (ODS) column (4 × 15 cm) chromatography and eluted MeOH-H_2O (3:2, 3.5 L) to afford 5 fractions (PSB1-1 to PSB-1–PBS-5) along with sakurasosaponin at PSB-1–PBS-4 (7.1 g).

In vitro cell death assays

PCa cells were treated with S-saponin at the dose or time indicated in the figures. Similarly, for the inhibitor experiment, PCa cells were cultured in the presence or absence of NAC to confirm the effect of frugoside. Cell death was measured by fluorescence-activated cell sorting (FACS) analysis after staining using an annexin V-fluorescein isothiocyanate/propidium iodide (annexin V-FITC/PI) staining kit (Roche, Nutley, NJ, USA). Cell viability was determined using a CCK-8 assay kit (Dojindo, Tokyo, Japan), according to the manufacturer's instructions. Briefly, PCa cells were inoculated into a 96-well microplate, and CCK-8 solution (10 μL/100 μL medium) was added to each well of the plate. After incubation of the plate for 1–4 h in a CO_2 incubator at 37 °C, the absorbance of each well was measured at 450 nm using a microplate reader (Molecular Devices, Sunnyvale, CA) with a reference wavelength of 650 nm. Cell cycle distribution was determined by DNA staining with PI (Sigma). Cells were harvested and fixed in 70% ethanol. Cell pellets were suspended in PI and simultaneously treated with RNase at 37 °C for 30 min. The percentage of cells in different phases of the cell cycle was measured using a FACScanto II flow cytometer (BD Biosciences).

Quantitative reverse transcriptase polymerase chain reaction

RNA was extracted from the cells using an RNeasy Mini kit (Qiagen). Briefly, 1.5 μg RNA was reverse transcribed with oligo (dT)12-18 primers using the First-Strand cDNA Synthesis Kit (Fermentas, Grand Island, NY, USA). All reactions were performed in triplicate, and the B2M gene was used as the control. Using the comparative threshold cycle (Ct) method or standard method, the relative quantification of expression of each gene was calculated after normalization against B2M for each sample. The primers for real-time PCR were designed as follows: AR primer: forward 5′-CCA AGC GCT AGT GTT CTG TTC-3′ and reverse 5′-GCA GCT TTT CTT GGG TCT CTT-3′; PSA primer: forward 5′-TGCCCACTGCATCAGGAACA-3′ and reverse 5′-GTCCAGCGTCCAGCACACAG-3′; TMPRSS2 primer: forward 5′-ATT TGC GGG GAT TTT GAG AC-3′ and reverse 5′-TCA ATG AGA AGC ACC TTG GC-3′; NKX3-1 primer: forward 5′-AAG CTC ACG GAG ACC CAA GT-3′ and reverse 5′-ATA CAC GGA GAC CAG GGA GG-3′; and B2M primer: forward 5′-CTC GCT CCG TGG CCT TAG-3′ and reverse 5′-CAA ATG CGG CAT CTT CAA-3′.

Protein isolation and Western blotting

Cells were lysed in lysis buffer A [20 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, 2 mM EGTA, 1% Triton X-100, 10% glycerol, and protease cocktail I/II; Sigma], and cellular debris was removed by centrifugation at 10,000×*g* for 10 min. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes, blocked with 5% skim milk in 0.01 M TBS (pH 7.5) containing 0.5% Tween 20, and blotted with the appropriate primary antibodies. The antigen–antibody complexes were detected by chemiluminescence (Abclone, Korea).

Measurement of mitochondrial activity

To detect and measure mitochondrial membrane potential, reactive oxygen species (ROS) generation, and Ca^{2+} concentration, we used the specific fluorescent probes TMRE, Mito-Sox, and Rhod-2AM (Invitrogen), respectively. Cells were cultured and then incubated with 1 μM Mito-Sox for 20 min or with 5 μM TMRE or Rhod-2AM for 30 min at 37 °C. The levels of the fluorescent probes were measured using a FACScantoII flow cytometer (BD Bioscience) and fluorescence microscope (Carl Zeiss, Germany).

Colony-forming assay

Anchorage-independent growth was assessed by performing colony-forming assays in soft agar assays. Cells were suspended in 1 ml cell growth medium containing 0.3% agar and plated over a layer of 0.6% agar in growth medium. Cells were grown at 37 °C with 5% CO_2. Fifteen days post-inoculation, the colonies were stained with 0.01% crystal violet for 10 min and counted.

Subcellular fractionation

For the mitochondrial leak experiments, the cytosolic and mitochondrial fractions were measured using a Proteo-Extract subcellular proteome extraction kit (Calbiochem, USA). Briefly, melanoma cells (1×10^6) were harvested, rinsed twice with ice-cold PBS, and resuspended in 200 μL of extraction buffer 1 containing a protease inhibitor cocktail (PIC). Subsequently, they were incubated with gentle agitation at 4 °C for 10 min. The supernatants were separated into pellets and supernatants (cytosolic fraction) by centrifugation at 1000×*g* for 10 min. The pellets were resuspended in 200 μL of extraction buffer 2 containing PIC, incubated with gentle agitation at 4℃ for 30 min, and then separated into pellets and supernatants (mitochondrial fraction) by centrifugation at 6000×*g* for 10 min.

Animal model

Tumorigenicity was determined by subcutaneously injecting 22Rv1 and PC-3 PCa cells into the flanks of 6-week-old

female nude mice (Japan). All mice were maintained in accordance with the institutional guidelines of the University of Ulsan Animal Care Committee. The tumor size was measured every 3 days using a digital caliper. The tumor volumes were measured using the formula $V = a \times b^2/2$, where a and b are the largest and the smallest superficial diameters of the tumor, respectively. At day 36, the tumor masses extracted from each group of mice were photographed.

To construct the prostate orthotopic xenograft model with PCa 22Rv1 cells, mice (8-week-old non-obese diabetic/severe combined immunodeficient mice (NOD. CB17-Prkdcscid)) were anesthetized using inhaled isoflurane, and a small nick was made in the skin. The prostate was exteriorized and injected with 200,000 22Rv1 cells in a collagen-1/PBS suspension. The mouse abdominal wall was closed, and the mouse was allowed to recover from anesthesia. The mice were euthanized at 5 weeks posttransplantation to assess tumor burden.

Statistics analysis

Data were analyzed using Student's *t* test with SigmaPlot 12.0 software (2013, Systat Software Inc., San Jose, CA, USA). The *P* values were derived to assess the statistical significance and are indicated as follows: $*P < 0.05$; $**P < 0.01$; and $***P < 0.001$. Data for all figures are expressed as the mean ± SD of three independent experiments.

Results

Identification of S-saponin as an inhibitor of PCa cells

In order to find natural compounds with anti-proliferative activity in PCa cells, we established a cell-based apoptotic assay using MR(DEVD)2 dye, which turns red upon caspase-3 cleavage in apoptotic cells. The hit compounds were further analyzed for PARP cleavage by Western blotting. The screening strategy scheme is depicted in Supplementary Fig. 1. We screened 400 natural compounds in our library, and 22 compounds induced apoptosis in LNCaP cells. Among 22 positive hits, four were triterpenoids. Supplementary Fig. 2A depicts the chemical structures of the representative triterpenoids. To compare the apoptosis activity, we treated these compounds (10 µg/ml) with LNCaP for 24 h. Among the four triterpenoid relative compounds, sakurasosaponin (S-saponin) displayed the most robust apoptotic activity (Supplementary Fig. 2B and C). Thus, we selected S-saponin as the most promising anti-proliferative agent to further examine in PCa cells.

S-saponin induces apoptosis in AR-positive PCa cells

To verify the inhibitory effects of S-saponin, cell proliferation was analyzed in several PCa cell lines, including LNCaP, 22Rv1, C4-2 (LNCaP cell derivative androgen-independent PCa), PC3, and DU145 cells. Cells were treated with various concentrations of S-saponin for 12–48 h, and their proliferation was determined by a CCK-8 cell viability assay. Pathologically different types of PCa cells were assessed, including LNCaP, 22Rv1, and C4-2, which contain AR, and PC3 and DU145, which are AR-null. LNCaP, 22Rv1, and C4-2 cells were relatively sensitive to S-saponin treatment (Fig. 1a–c). However, the growth and cell death of PC3 and DU145 cells were not affected by treatment with S-saponin (Supplementary Fig. 3A–D). To further explore the effect of S-saponin on PCa cell death, LNCaP, 22Rv1, and C4-2 cells were treated with S-saponin, labeled with annexin V-FITC and examined by flow cytometry. As shown in Fig. 1d–f, S-saponin induced cell death in a concentration-dependent manner. To confirm whether the cell death induced by S-saponin was due to apoptosis, we measured the DNA content of LNCaP, 22Rv1, and C4-2 cells after S-saponin treatment. S-saponin treatment increased the sub-G1 population in a dose-dependent manner (Fig. 1g–i). Collectively, these results support that S-saponin regulates cell growth and cell death in AR-positive (androgen-dependent and castration-resistant) PCa cells.

S-saponin induced mitochondria-mediated PCa cell death

Since caspases are major apoptotic regulators, we investigated caspase activation in S-saponin-treated LNCaP and 22Rv1 cells. S-saponin induced caspase-3 and PARP cleavage in a time- and dose-dependent manner (Fig. 2a–d), confirming the cytotoxicity of S-saponin. We further confirmed that stout activation of caspase-3 and DNA fragmentation were demonstrated in these cell lines (Fig. 2e and f). We next examined potential mitochondrial involvement in cell death mediated by S-saponin. As shown in Fig. 2g and h, cytochrome c release into the cytosol was markedly increased by concentration-dependent treatment with S-saponin. Next, PCa cells were treated with S-saponin to evaluate potential differences in mitochondrial function in cells. FACS analysis showed that S-saponin had decreased mitochondrial membrane potential; however, mitochondrial ROS and Ca^{2+} levels were higher in S-saponin treatment than in control groups in both LNCaP and 22Rv1 cells (Fig. 3a and b). We confirmed the alteration of mitochondrial function by S-saponin using fluorescence microscopic analysis, with similar observations (Fig. 3c–h). Collectively, these results suggested that the anti-PCa effects of S-saponin are due to mitochondrial-mediated apoptotic cell death.

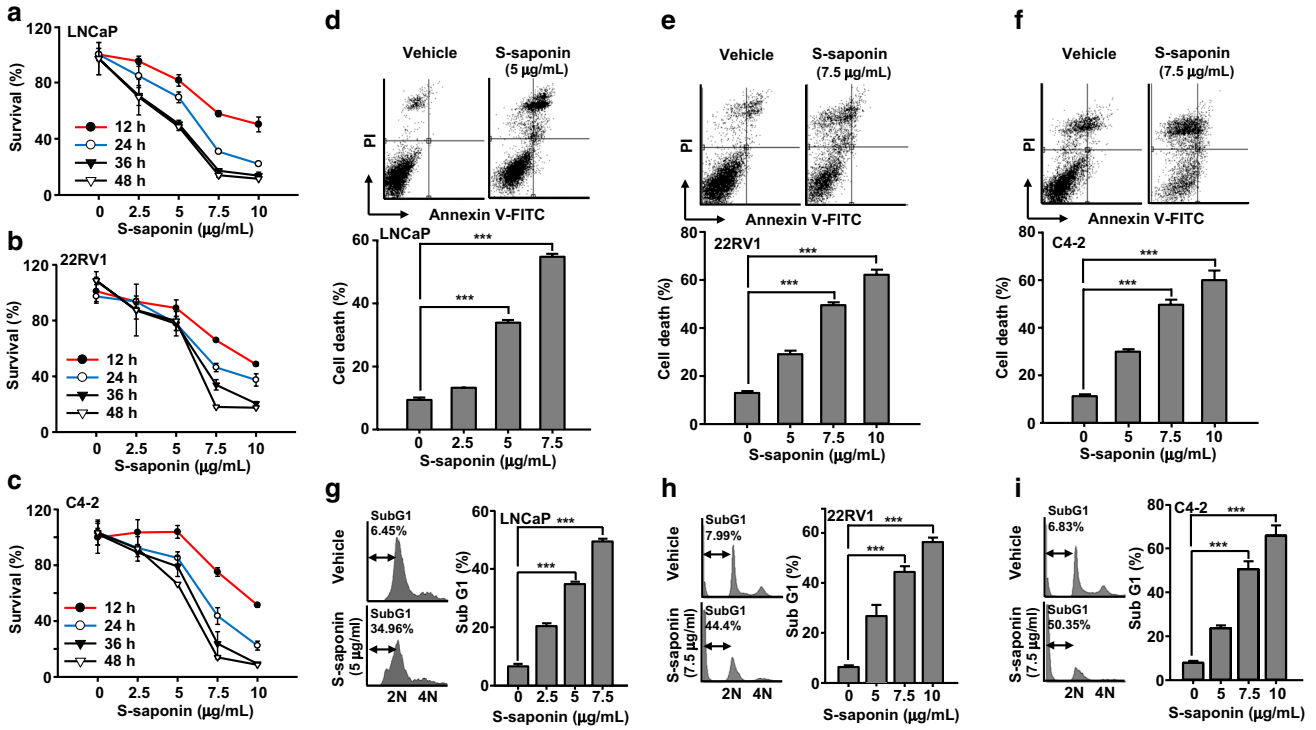

Fig. 1 S-saponin induces cell death in androgen-dependent and castration-resistant prostate cancer cells. **a**–**c** LNCaP (**a**), 22Rv1 (**b**), and C4-2 (**c**) cells were treated with S-saponin according to the indicated dose and time. The level of cytotoxicity was measured by the CCK-8 assay. **d**–**i** LNCaP, 22Rv1, and C4-2 cells were treated with S-saponin at the indicated dose for 24 h. The cell viability of S-saponin-treated LNCaP (**d**), 22Rv1 (**e**), and C4-2 (**f**) cells was determined by FACS analysis after annexin V-FITC/PI staining. The apoptotic cell population (Sub G1 population) was measured by PI staining and FACS analysis in S-saponin-treated LNcap (**g**), 22Rv1 (**h**), and C4-2 (**i**) cells

S-saponin inhibits AR expression and its target genes

AR is a critical factor for cell survival and growth in PCa [10]. Thus, considering that S-saponin-mediated cell toxicity in AR-positive cells is related to modulation of AR by S-saponin, we assessed whether S-saponin affects AR expression. LNCaP, 22Rv1, and C4-2 cells treated with different doses of S-saponin for different times and cell lysates were prepared, followed by Western blot analysis. We observed dose- and time-dependent decreases in AR protein levels with S-saponin treatment in three cell lines: LNCaP, 22Rv1, and C4-2 (Fig. 4a, b). Furthermore, to determine whether a decrease in AR protein caused by S-saponin occurs at the posttranslational level, we examined the half-life of the protein level of AR in the presence of cycloheximide. As shown in Fig. 4c, there was no difference in AR stability between vehicle-treated cells and S-saponin-treated cells. Similarly, MG132 failed to restore the AR protein in LNCap cells after treatment with S-saponin (Fig. 4d). Thus, we next assessed the mRNA levels of AR in LNCaP and 22Rv1 cells and found that they were altered by S-saponin treatment (Fig. 4e, f). Together, these data indicate that S-saponin-induced downregulation of AR is the result of decreased transcription. We next determined whether the inhibition of AR expression by S-saponin regulates the transcriptional activity of AR. LNCaP and 22Rv1 cells were treated with vehicle or S-saponin for various times and doses, and then, we measured AR target genes including prostate-specific antigen (PSA), transmembrane protease, serin 2 (TMPRSS2), and NK3 homeobox 1 (NKX3.1). S-saponin treatment decreased transcription of AR target genes in LNCaP and 22Rv1 cells (Fig. 5a–d). We also tested the protein levels of AR target genes in LNCaP and 22Rv1 cells treated with S-saponin at different doses and times, and as expected, S-saponin significantly inhibited the expression of TMPRSS2 and NKx3.1 (Fig. 5e, f).

Anti-apoptotic protein Bcl-xL as an AR target gene is involved in S-saponin-mediated cell death

Having shown the mitochondrial involvement in S-saponin-induced cell death, we also tested the transcription of the gene Bcl-xL, which is known to be regulated by AR [22, 23]. As shown in Fig. 6a and b, the protein and mRNA levels of Bcl-xL were significantly decreased in response to S-saponin treatment in both 22Rv1 and C4-2 cells. To confirm that S-saponin-induced cell death occurs through

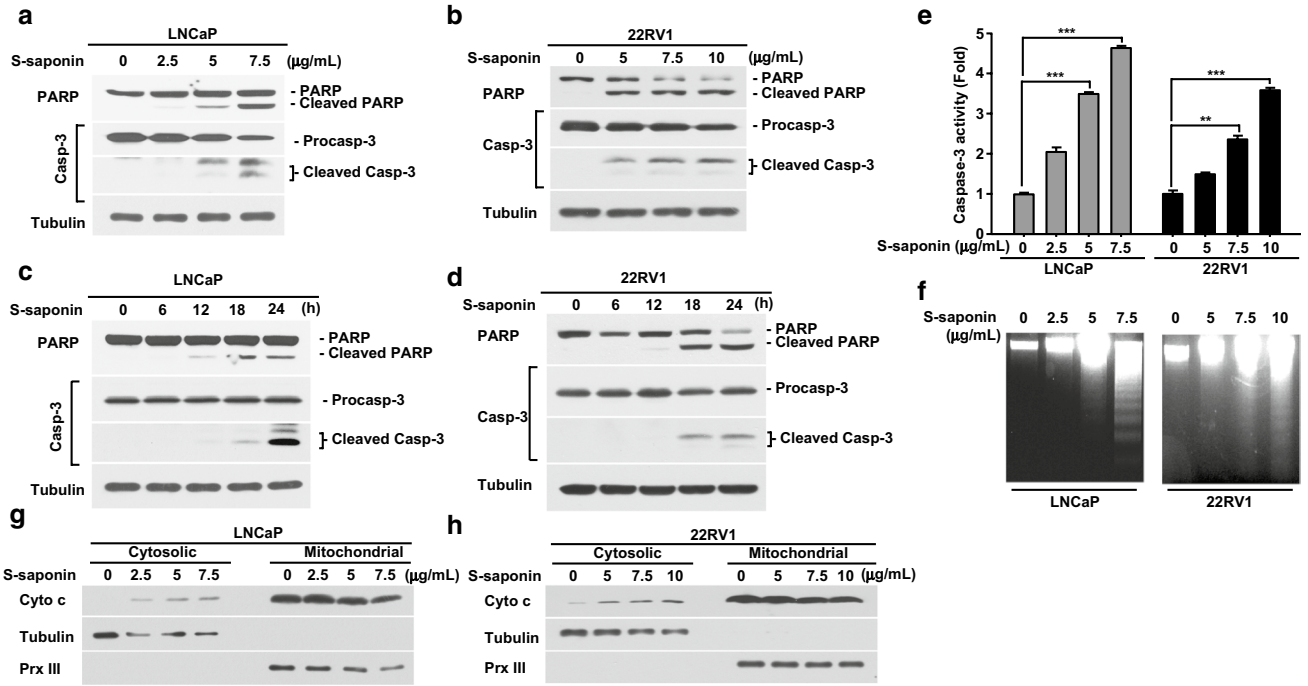

Fig. 2 Effect of S-saponin on caspase activation indicating apoptotic cell death of prostate cancer cells. **a**–**d** LNCaP and 22Rv1 cells were treated with S-saponin in a dose-dependent manner for 24 h (**a**, **b**) and in a time-dependent manner (**c** 5 μg/ml, **d** 7.5 μg/ml). The cells were subjected to Western blotting using the indicated antibodies. **e** LNCaP and 22Rv1 cells were treated with S-saponin in a dose-dependent manner, and caspase-3 activity was measured. **f** DNA fragmentation was measured in the S-saponin-treated LNCaP and 22Rv1 cells via agarose gel separation after staining with EtBr. **g**, **h** LNCaP and 22Rv1 cells were treated with S-saponin for 24 h, at the indicated doses, and the cells were separated into cytosolic and mitochondrial fractions. Tubulin and Prx3 were used as cytosolic and mitochondrial markers, respectively

a Bcl-xL-dependent mechanism, we transiently transfected 22Rv1 cells with Bcl-xL plasmid and then treated 22Rv1 cells with or without S-saponin. S-saponin elicited robust cell death, which was significantly inhibited by BCL-xL transfection (Fig. 6c), suggesting that S-saponin-induced cell death occurs through a Bcl-xL-dependent mechanism. We further confirmed whether overexpression of AR plasmid affected the S-saponin-induced cell death in 22Rv1 cells. 22Rv1 cells were transfected with AR-overexpressing or control plasmid for 24 h and then treated with S-saponin for 24 h. As shown in Fig. 6d, AR-transfected cells were more resistant to S-saponin-induced cell death than control plasmid-transfected 22Rv1 cells. Notably, compared with the control, the amount of the endogenous BCL-xL was markedly increased in AR-overexpressed cells and S-saponin-treated AR-overexpressed cells. Moreover, S-saponin-induced cleavage of caspase-3 and PARP was suppressed in Bcl-xL or AR-transfected 22Rv1 cells (Fig. 6e, f). Next, we determined if S-saponin-mediated decreased BCL-xL expression was involved with oxidative stress. To determine whether S-saponin-mediated decreased BCL-xL expression is involved with oxidative stress, we pretreated 22RVI cells with *N*-acetylcystein (NAC), a known ROS scavenger, followed by S-saponin treatment. NAC attenuated S-saponin-induced cytotoxicity in 22Rv1 cells (Fig. 6g). In addition, co-treatment with NAC inhibited the cleavage of caspase-3 and PARP by S-saponin in 22Rv1 cells (Fig. 6h).

Hence, S-saponin-induced cell death presumably occurred via inhibition of BCL-xL expression and induction of oxidative stress.

S-saponin-induced AR deficiency suppresses tumor growth in vivo

To evaluate the in vivo effects of S-saponin, we first used an anchorage-independent growth assay using sphere formation. As shown in Fig. 7a, the number of spheres showed a significant concentration-dependent decrease after treatment with S-saponin. Next, we used a xenograft model produced by the injection of 22Rv1 and PC-3 cells. In 22Rv1 xenograft-bearing mice, S-saponin injection resulted in a greatly reduced tumor volume compared with the vehicle (Fig. 7b). To further confirm this finding, we used an orthotropic model of 22Rv1 cells injected into the anterior prostate lobes of SCID mice. The results indicated that S-saponin notably decreased tumor volumes by 52%. Interestingly, the tumor growth rate change caused by S-saponin was not observed in PC-3 xenograft mice (Fig. 7c). Furthermore, qRT-PCR

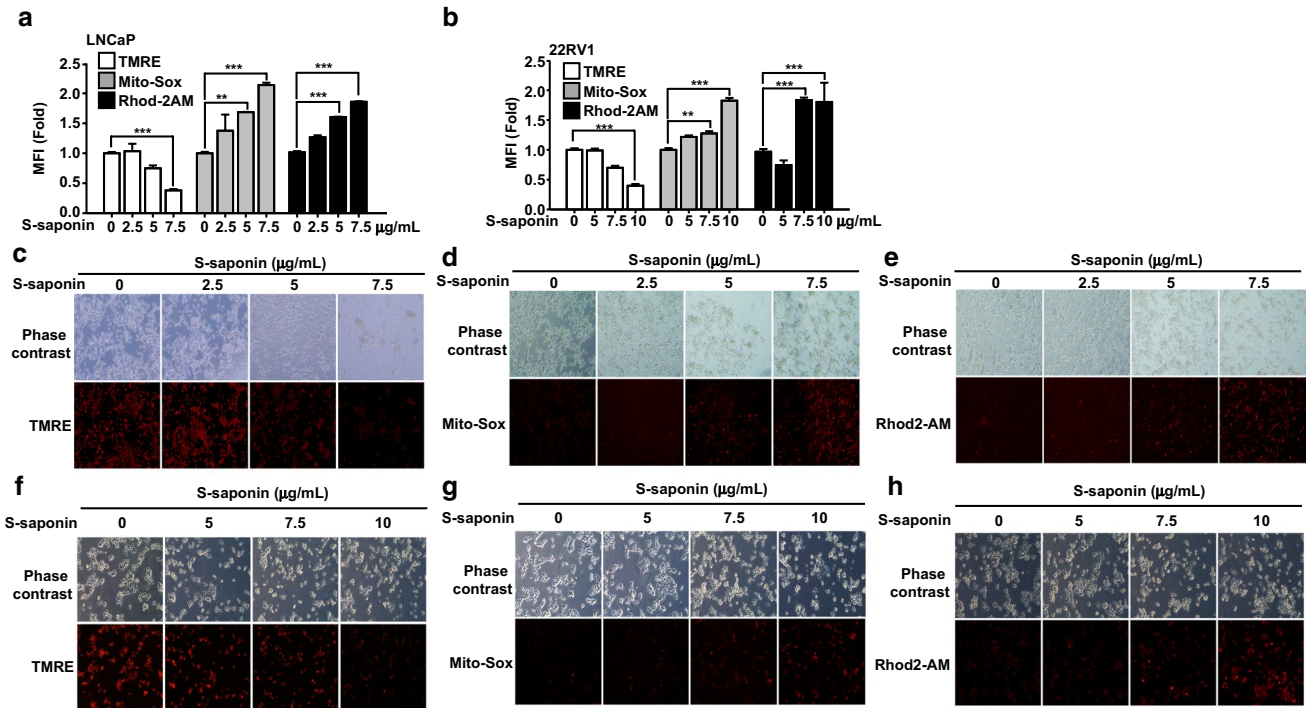

Fig. 3 S-saponin treatment results in mitochondrial dysfunction via ROS overproduction. **a**, **b** LNCaP and 22Rv1 cells were treated with S-saponin at the indicated doses for 24 h, and the mitochondrial function was measured by examining mitochondrial membrane potential (TMRE), mitochondrial ROS level (Mito-Sox), and mitochondrial calcium level (Rhod2-AM) using a FACScanto II. (C–E) LNCaP cells were treated with S-saponin in a dose-dependent manner for 24 h and analyzed using a fluorescence microscope after staining with TMRE (**c**), Mito-Sox (**d**), and Rhod2-AM (**e**). **f**–**h** 22Rv1 cells were treated with S-saponin at the indicated doses for 24 h and analyzed using a fluorescence microscope after staining with TMRE (**f**), Mito-Sox (**g**), and Rhod2-AM (**h**)

and immunoblotting results showed that AR expression was significantly decreased in the tumors treated with S-saponin, compared to the untreated tumors (Fig. 7d, e). Collectively, these data show that S-saponin treatment reduces the tumorigenic ability in vivo through inhibiting AR expression in PCa cells.

Discussion

PCa accounts for 26% of all cancers in men in the USA and is one of the most common cancers worldwide [1, 2]. Most PCs are hormone dependent, and androgen is important in the progression of PC growth. In classical AR signaling, androgen activates the transcriptional activity of the AR by binding with the AR and mediates the growth of PCa cells [10]. For patients, hormone therapy, which inhibits ligand binding to receptors and the activity of AR by androgen blockade, is successful at first but ultimately fails to treat the disease [6, 27]. The main mechanism for progression to castration-resistant (androgen-independent) PCa is due to the reactivation of the AR [9, 13, 28]. Thus, regulation of AR expression and the transcriptional activity is important in androgen-dependent PCa, as well as with castration-resistant metastatic PCa with resistance to androgen deprivation therapy.

Many types of natural compounds in foods, herbs, or plants have been reported to have anticancer effects on various types of cancer [29, 30]. S-saponin, a newly identified saponin known to inhibit the growth of various human cancer cells (breast cancer, lung cancer, colon cancer, and melanoma), is derived from the leaves of *Rapanea melanophloeos* and *Aegiceras corniculatum*, stem bark of *Tapeinosperma clethroides,* and roots of *Jacquinia flammea* Millsp; however, its exact anticancer mechanism is unknown.

In the present study, through screening a library of natural compounds, we obtained a few potent natural compounds that efficaciously promote the death of PCa cells, and S-saponin was among them. We have determined the effects of S-saponin on the expression of AR and growth of human PCa cells. S-saponin significantly inhibited the expression of AR and abrogated the activation of AR in both androgen-dependent (LNCaP) and castration-resistant (22Rv1 and C4-2) PCa cells. In addition, Bcl-xL expression is dependent on the expression of AR, and inhibition of AR expression by S-saponin decreases Bcl-xL expression. Moreover, S-saponin-mediated AR inhibition induces cell death through the mitochondrial-mediated apoptotic pathway, and

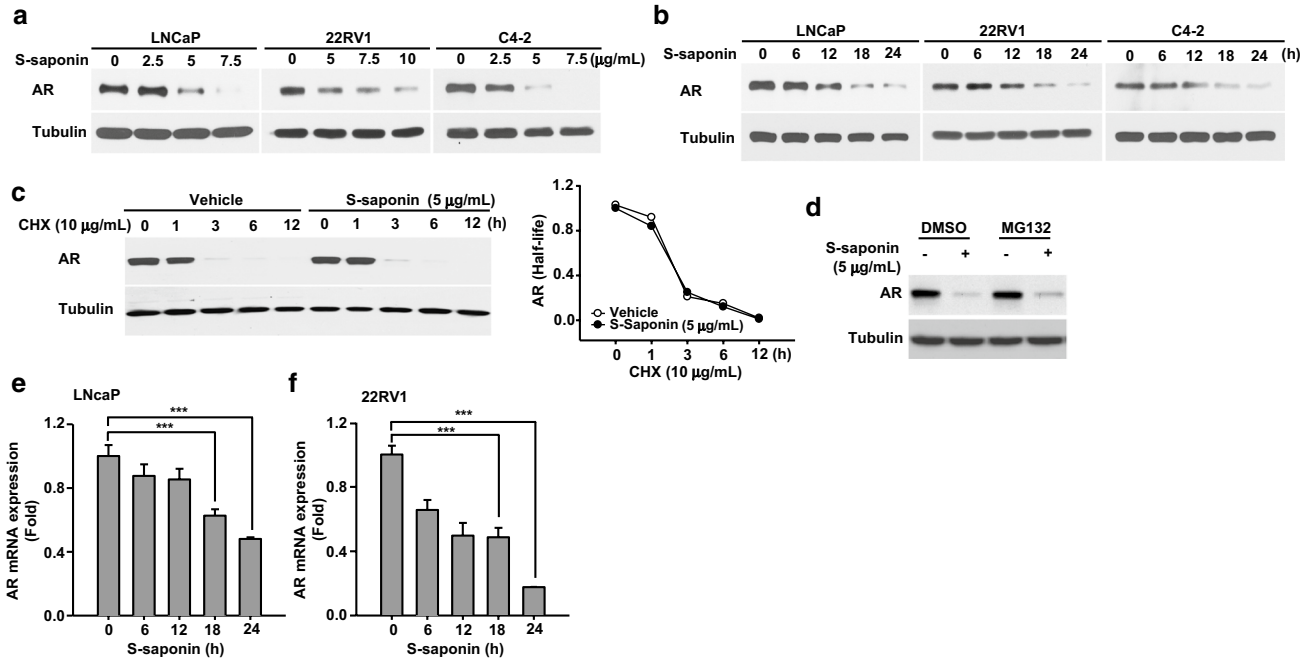

Fig. 4 S-saponin induces a reduction in AR expression in prostate cancer cells. **a**, **b** LNCaP, 22Rv1, and AIPC cells were treated with S-saponin in a dose-dependent manner (**a**) for 24 h. LNCaP (0.5 μg/ml), 22Rv1 (7.5 μg/ml), and C4-2 (7.5 μg/ml) cells were treated with S-saponin in a time-dependent manner (**b**). The treated cells were subjected to Western blotting. **c** LNCaP cells were treated with S-saponin for the indicated times after 1 h cycloheximide pretreatment and subjected to Western blotting. The AR band intensity was measured and is represented in the graph (right panel). **d** LNCaP cells were treated with S-saponin for 12 h after 1 h MG132 pretreatment and subjected to Western blotting. **e**, **f** LNCaP (**e**) and 22Rv1 (**f**) cells were treated with S-saponin in a time-dependent manner. The mRNA expression of the cells was measured using qPCR

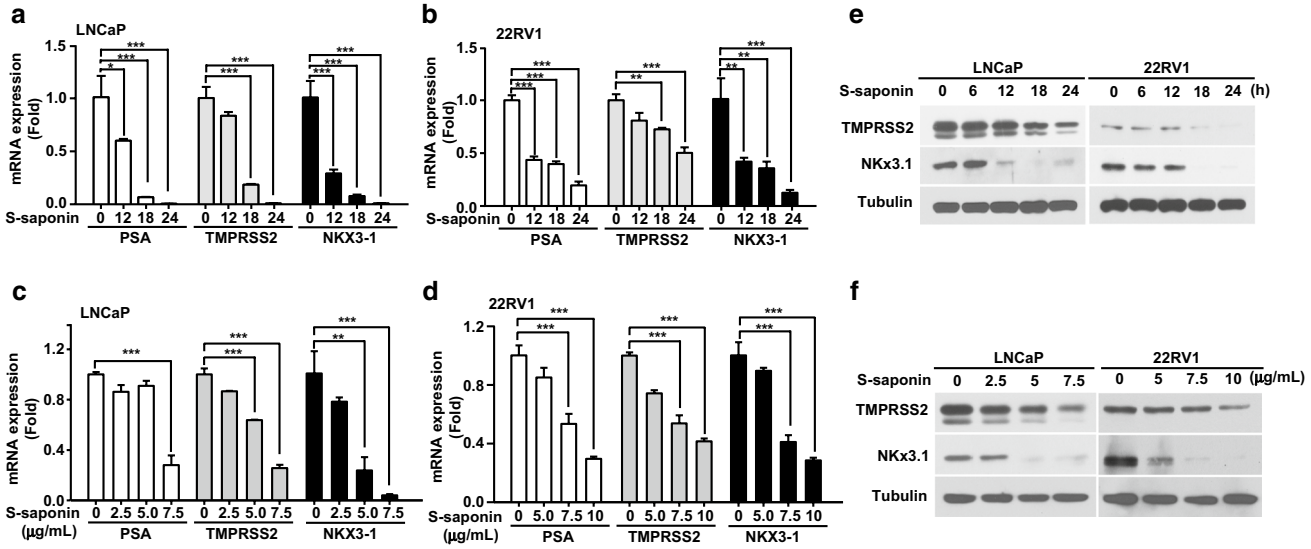

Fig. 5 Reduction in AR by S-saponin treatment results in the inhibition of AR target gene expression. **a**–**c** LNCaP (**a**; 5 μg/ml) and 22Rv1 cells (**b**; 7.5 μg/ml) were treated with S-saponin for the indicated times. The cells were subjected to qPCR analysis to measure the expression of AR target genes, PSA, TMPRSS2, and NKX3-1, respectively. The translational expression of AR target genes was measured by Western blotting (**c**) with antibodies against TMPRSS2 and NKx3-1. **d**–**f** LNCaP cells (**d**) and 22Rv1 cells (**e**) were treated at the indicated dose for 24 h and subjected to qPCR and Western blotting analysis (**f**), respectively

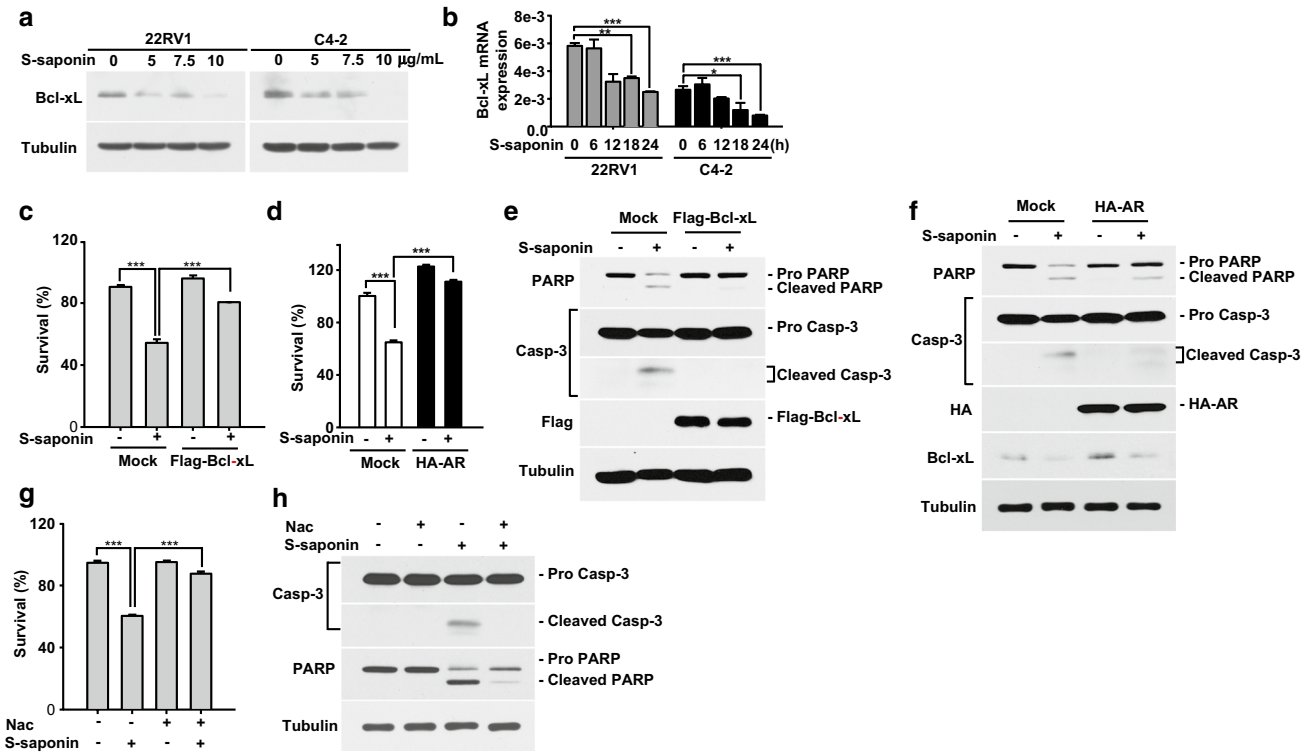

Fig. 6 Reduction in AR by S-saponin induces cell death via a decrease in Bcl-xL protein and sequential mitochondrial ROS overproduction in PCa cells. **a** 22Rv1 and C4-2 cells were treated with S-saponin in a dose-dependent manner, and the Bcl-xL protein level was measured by Western blotting with Bcl-xL and tubulin antibodies. **b** 22Rv1 cells and C4-2 cells were treated with 7.5 μg/ml S-saponin in a time-dependent manner, and the transcriptional expression of the Bcl-xL gene was measured using qPCR. **c** 22Rv1 cells were transfected with mock or flag-Bcl-xL plasmids, and the cells were treated with 7.5 μg/ml s S-saponin for 24 h. Cell death was measured by FACS analysis after staining with annexin V-FITC and PI reagent. **d** 22Rv1 cells were overexpressed with AR plasmids or mock and treated with 7.5 μg/ml S-saponin for 24 h. **e**, **f** 22Rv1 cells were transfected with flag-Bcl-xL (**e**) and HA-AR plasmids (**f**), respectively. The cells were subjected to Western blotting with antibodies against PARP, caspase-3, flag, HA, tubulin, and Bcl-xL proteins. **g**–**h** 22Rv1 cells were treated with 7.5 μg/ml S-saponin 24 h later for 1 h pretreatment with NAC (10 mg/ml). The cells were subjected to FACS analysis (**g**) after staining with annexin v-FITC/PI reagents and analyzed by Western blotting (**h**) with antibodies against caspase-3, PARP, and tubulin proteins

such apoptotic cell death is only observed in AR-expressing PCa cells regardless of their androgen sensitivity. Thus, this finding provides a molecular mechanism for how S-saponin induced mitochondrial-mediated cell death in androgen-dependent (LNCaP) and castration-resistant (22Rv1 and C4-2) PCa cells.

Bcl-xL is an anti-apoptotic protein that modulates mitochondrial permeability and subsequently regulates the intrinsic apoptosis pathway [31, 32]. Through downregulation of Bcl-xL, the permeability of the outer membrane of mitochondria can promote the release of mitochondrial cytochrome c into the cytoplasm [33–35]. Bcl-xL, also known as the direct target gene of AR, has been reported to be essential for proliferation of AR-positive PCa cells [22, 23]. Recently, reports have suggested that the silencing of BCL-xL induces apoptosis in LNCaP and reduces tumor growth in vivo [36–38]. A previous study had revealed that inhibition of BCL-Xl-signaling by using BCL2 specific inhibitor can sensitize CRPC to enzalutamide [39]. Therefore, we suggest that S-saponin is a good candidate agent for use in androgen receptor-dependent PCa treatment.

In this study, we provide a potential contributing mechanism to AR-associated PCa cell death. S-saponin-mediated inhibition of AR was associated with dramatic alterations in the expression of Bcl-xL and significant mitochondrial dysfunction. We also observed that S-saponin treatment led to a significant decrease in the mitochondrial membrane potential, increase in the release of cytochrome c into the cytosol, increase in the activity of caspase-3, and the induction of apoptosis. Presumably, these results support that S-saponin-induced apoptosis is mediated by the intrinsic mitochondrial pathway.

In the current study, our results show that S-saponin inhibits expression of AR at both the mRNA and protein levels and decreases cell growth of AR-positive PCa cells through regulation of AR target molecules, including

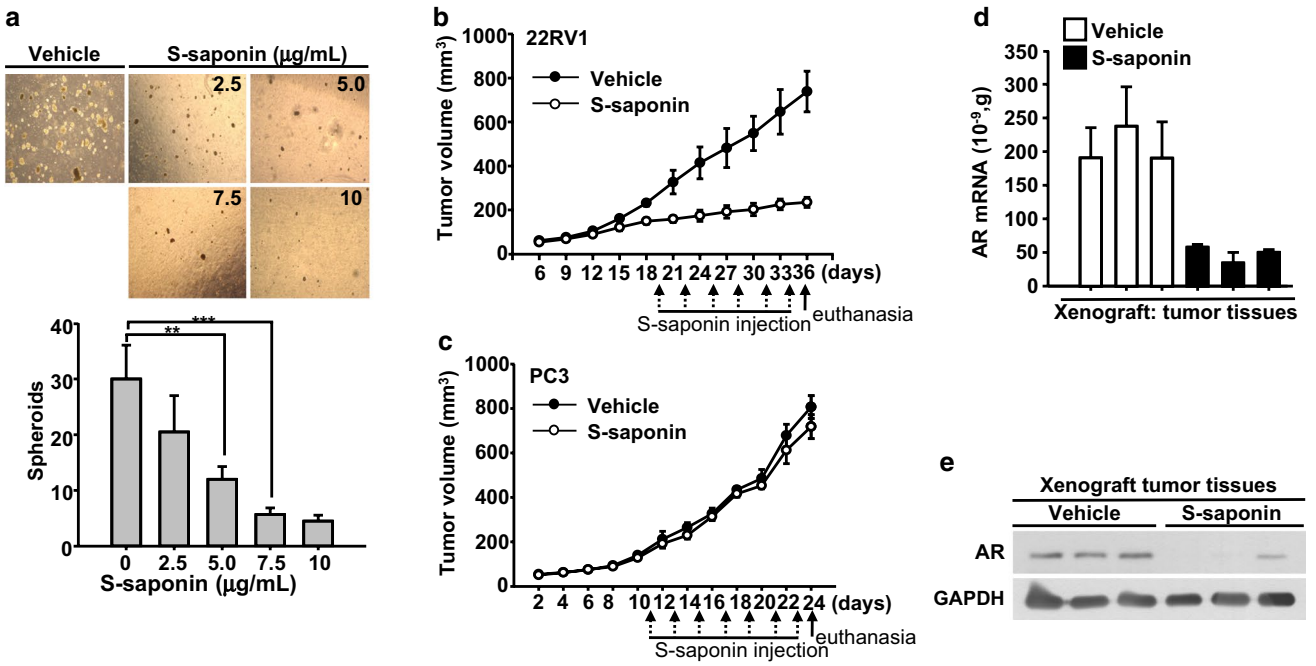

Fig. 7 S-saponin inhibits tumorigenic ability in vitro and in vivo. **a** 22Rv1 cells were plated for sphere formation assays in ultra-low attachment plates and treated with S-saponin in a dose-dependent manner. Spheroids generated per 10,000 cells were counted 2 weeks later. **b**, **c** To generate the xenograft model, 1×10^6 22Rv1 (**b**) and PC-3 (**c**) cells were subcutaneously injected into the flanks of 8-week-old male BALB/c nude mice. After 2 weeks, the mice were randomly divided into two groups of three (the average tumor volume was 70.1 mm^3). S-saponin was intraperitoneal administered at doses of 200 μg/kg once every 3 days for 20 days. Tumor volume was calculated using the formula $V=a\times b^2/2$, where a and b are the largest and the smallest superficial diameters of the tumor, respectively. At day 24 or 36, 22Rv1- and PC-3-inoculated mice were killed, and the tumor masses were extracted. **d**, **e** Extracted tumor masses (**b**) were subjected to qPCR (**d**) and Western blotting (**e**) to determine the level of AR expression, representing the effect of S-saponin

BCL-XL, PSA, TMPRSS2, and NKX3.1. In addition, S-saponin presented minimal toxicity in AR-negative PCa cells and significantly inhibited 22Rv1 PCa cell growth in the xenograft and orthoxenograft models. Overall, this study suggests that S-saponin could be a potent agent for the treatment of androgen sensitive, but most importantly, castration-resistant AR-expressing PCa. Although more mechanism studies are needed to determine the potential of S-saponin as an anticancer drug for PCa, we suggest that S-saponin is a useful agent for suppression of PCa progression in AR-positive human PCa.

Author contributions I-SS and S-WJ designed the research; K-HS and N-IB isolated and identified S-saponin; I-SS, YJJ, JEK, and JS performed the research; I-SS, YJJ, JEK, JS, and S-WJ analyzed the data; and I-SS and S-WJ wrote the paper.

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Compliance with ethical standards

Conflict of interest No potential conflicts of interest are disclosed.

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