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Pomegranate extracts impact the androgen biosynthesis pathways in prostate cancer models *in vitro* and *in vivo*

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ABSTRACT

Castration-resistant prostate cancer (CRPC) remains largely dependent on androgen receptor (AR). Residual tissue androgens are consistently detected within CRPC tumors and play a critical role in facilitating AR-mediated signaling pathways which lead to disease progression. Testosterone and dihydrotestosterone (DHT) are the major androgens detected in tumors. They are produced through three biosynthesis pathways: Δ^4, Δ^5, and backdoor pathways. Both androgens bind to and stimulate AR activation. The current study investigates the effects of pomegranate extracts (POM) and their ability to inhibit androgen biosynthesis using PCa cell lines (22RV1 and LNCaP) *in vitro* as well as the PTEN knockout mouse model representing prostate cancer. Steroids were extracted using ethyl acetate or solid phase extraction, and then analyzed by UPLC/MS/MS. The results showed that POM (0–12 μg/mL) reduced the production of testosterone, DHT, DHEA, androstenedione, androsterone, and pregnenolone in both cell lines. In addition our *in vivo* data supports this observation with a reduction in serum steroids determined after 20 weeks of POM treatment (0.17 g/L in drinking water). In accordance with these results, Western blotting of cell lysates and tPSA analysis determined that PSA was significantly decreased by the treatment of POM. Interestingly, AKR1C3 and AR levels were shown to be increased in both cell lines, perhaps as a negative feedback effect in response to steroid inhibition. Overall, these results provide mechanistic evidence to support the rationale for recent clinical reports describing efficacy of POM in CRPC patients.

1. Introduction

Prostate cancer (PCa) is a commonly diagnosed cancer in North American men and is one of the leading causes of cancer death in western countries [1]. Androgen deprivation therapy has been the primary treatment for patients with metastatic PCa. Although initially effective, most patients with PCa progress to castration-resistant disease over a period of 18–20 months [2,3]. Residual androgens detected within the prostate tumors in castrated patients are thought to be a driving factor in androgen receptor (AR) mediated signaling and gene expression, which lead to disease progression. The enzymes responsible for the biosynthesis of testosterone and dihydrotestosterone have been identified and their increased expression within castration resistant metastases implies that targeting those enzymes may be of benefit in advanced PCa treatment [4]. Several drug compounds are currently used to inhibit local steroidogenesis in the tumor e.g. abiraterone acetate (targeting CYP17) [5] and dutasteride (targeting 5-alpha reductase), and are used in conjunction with an AR antagonist such as MDV3100 [6,7].

Pomegranate extracts (POM) have been reported to be active in the treatment of parasitic and microbial infections, diarrhea, ulcers, hemorrhage, respiratory complications, and cardiovascular diseases [8–10]. The unique antioxidant tannins and flavonoids contained in POM have recently drawn the attention of many scientists and the biological activities of pomegranate-derived products, especially their anti-inflammatory and anticancer properties, are being investigated. Recent research has shown that POM selectively inhibit the growth of breast, prostate, colon and lung cancer in cell culture and animal models [11]. Pomegranate juice has shown an initial promise in a phase II clinical studies against PCa [12,13]. However, the effect of POM on steroid biosynthesis pathways have yet to be delineated. This study investigates the effects of POM on androgen biosynthesis pathways specifically using two PCa cell lines (22RV1 and LNCaP) as well as a murine PCa mouse model (conditional PTEN knockout prostate cancer model).

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2. Materials and methods

2.1. Chemicals and reagents

Dihydrotestosterone (DHT), testosterone, dehydroepiandrosterone (DHEA), androstenedione, androstanedione, pregnenolone, progesterone, hydroxyprogesterone, androsterone, 5α-pregnane-3, 17-diol-20-one, and 5α-pregnane-3, 20-dione were purchased from Steraloids (Newport, RI, USA). Internal standards (IS) testosterone-d_3 and DHT-d_3 were obtained from C/D/N isotopes (Pointe-Claire, QC, Canada). Standard compounds were stored according to supplier recommendations. Working stock solutions were prepared at the concentration of 1000 μg/mL and 20 ng/mL for standards and internal standards, respectively. Fetal bovine serum (FBS) and Charcoal stripped fetal bovine serum (CSS) was acquired from Hyclone (Logan, UT, USA) and was stored at −20 °C before use in the preparation of media. RPMI 1640 media were obtained from Life Technologies (Grand Island, NY, USA) and Hyclone (Logan, UT, USA). Optima grade acetonitrile, methanol, acetone, and ethyl acetate (Fisher, ON, Canada), were used for the preparation of mobile phase or extraction. LC/MS grade formic acid and hydroxylamine hydrochloride were purchased from Sigma-Aldrich Canada (Oakville, ON, Canada). 18.2 MΩ-cm (Meg ohm) ultra-pure water used was from Millipore Ultra-Pure Water System. Ultra-pure argon gas (argon 1006, >99.999%) was used as collision gas and was obtained from Praxair Canada Inc.

2.2. Cell culture

The LNCaP and 22RV1 human prostate cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). >Both cell lines were routinely cultured in the RPMI 1640 with L-glutamine and supplemented with 5% FBS. Cells were cultured at 37 °C and 5% CO_2 in an incubator, and the media were changed every 48 h. When cultures grew to 70% confluency in the 15 cm (diameter) plate, the media were aspirated and the cells were washed with phosphate buffer solution (PBS), for further culturing in the either cell control media or cell treatment media (containing POM) for 48 h. The final culture time was optimized by testing steroid concentrations after three culturing time periods: 24, 48, and 72 h. The LC/MS/MS results indicated that the steroid concentrations in the media reach their peak values at 48 h therefore the 48 h time point was selected for the purpose of our experiments.

2.3. Pomegranate extracts

Pomegranate extracts were provided by Verdure Sciences Inc (Noblesville, IN, USA). Whole pomegranate fruit was broken into small pieces and extracted using 100% ethanol. The ethanol solution was evaporated and lyophilized. The dried powder was re-dissolved in ethanol, and then centrifuged at 20,000 rcf for the separation of ethanol soluble fraction and the water soluble residue. The residue was dried and reconstituted in ethanol–water (70:30) mixture to form a solution of 200 mg/mL, which was applied to prepare the serial cell treatment media used in the experiment. The main chemical constituents of the water soluble extracts were identified using LC/MS and compared with the authentic standards.

2.4. Cell treatment with POM

Two different precursor steroids, progesterone or DHEA, were used to evaluate steroidogenesis and each was supplemented into the cell culture media in two separate experiments. The control media were prepared using RPMI 1640 with 5% CSS, and supplemented with 2 μg/mL of progesterone or DHEA. In the DHEA treated group, both cell lines LNCaP and 22RV1 were used for the experiments. Cell treatment media was prepared with RPMI 1640, 5% CSS, DHEA (2 μg/mL), and pomegranate water soluble extracts (POM). The serial concentrations of POM in the four treatment groups were 2 μg/mL, 5 μg/mL, 8 μg/mL, and 12 μg/mL, respectively. For the progesterone treated group, only the 22RV1 cell line was used. The cell treatment media used with 22RV1 cells for the progesterone group were prepared in the same way as the DHEA group but only one concentration of POM (12 μg/mL) was used to treat the cells. After the cells were cultured with the control or treatment media for 48 h, the media were collected, and the cells were harvested using a plastic scraper and transferred into Eppendorf micro centrifuge tubes. The cells pellets were weighed and stored at −80 °C before analysis. All of the cells and media from controls and POM treated samples were analyzed for steroids using UPLC–MS/MS after suitable extraction.

2.5. Cell viability assays

Cell viability assays were carried out using tetrazolium [3-(4,5-dimethylthiazol-2-yl)–5-(carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazoilium, inner salt (MTS, Promega, Madison, USA) and Trypan blue. Briefly, cells were initially cultured in 96 well plates (Corning, NY, USA) and each well contained 2000 cells in 100 μL of media (RPMI 1640 with 5%FBS). After 48 h, the cells were treated with serial concentrations of POM (0, 4, 8, 12, 16, 20, 100, 250, 500, and 1000 μg/mL) prepared in RPMI 1640 with 5% CSS. The cells were continued to be cultured 48 h and then mixed with MTS or Trypan blue for the viability assay. MTS assay was performed by adding 10 μL of MTS solution directly to the culture wells, incubating 1 h and then recording the absorbance at 490 nm using a 96-well plate reader. Trypan blue is prepared as 0.4% solution with PBS. Twenty microliter of cell suspension was mixed with 20 μL Trypan blue solution and then 10 μL of mixture was added to each side of the dual chamber on a Bio Rad TC10 TM system counting slide. Then the live and dead cell numbers were countered using Bio Rad TC10 Automated cell counter. Cell viability was calculated as the number of viable cells divided by the total number of cells.

2.6. Instrumentation

A Waters ACQUITY Quattro Premier UPLC/MS system (Waters Mississauga, ON, CA), consisting of a sample manager, a binary solvent manager, a column manager, a PDA detector, and a triple quadrupole (Quattro Premier XE) mass detector, was used in this investigation. MS control and spectral processing were carried out using Masslynx software, version 4.1 (Waters).

2.7. UPLC–MS/MS conditions

The analytes were separated on a Waters ACQUITY UPLC system using a 2.1 mm × 100 mm × 1.7 μm ACQUITY BEH C18 column with a flow rate of 0.3 mL/min. Mobile phase A and B were water and acetonitrile, respectively, and both contained 0.1% formic acid. A gradient method was used and the percentage of organic solvents was changed as follows: 0.00 min, 25%; 5 min, 70%, 7 min 100%, 10 min, 100%, 10.1 min, 25%, 12 min, 25%. Strong wash solution consisted of isopropanol, methanol, and formic acid (50:50:0.2) and weak wash solution was methanol and water (20:80). Ten microliters of each sample were injected into the column. The column and autosampler temperatures were held at 35 and 20 °C, respectively.

Mass spectrometry was performed using Quattro premier XE detector equipped with electrospray ionization source (ESI). Positive ionization was used in the multiple reaction monitoring (MRM) mode, with two transitions for each compound. The capillary voltage was set to 1.0 kV. The source and desolvation temperatures

were optimized and kept at 120 and 350 °C, respectively. The desolvation gas was delivered at a flow rate of 800 L/h and the cone gas was set to 50 L/h. The pressure of collision gas (Argon) was maintained at 7 psi. The appropriate MRM transitions, cone voltages, and collision energies were obtained by infusion (10 μL/min) and LC flow (0.3 mL/min) into the mass spectrometer together.

Basal levels of steroids in cells and media are inherently low and can hardly be detected by LC/MS/MS. Hydroxylamine derivatives have typically been used to improve sensitivity for analysis [14,15]. Hydroxylamine reacts with the carbonyl group(s) of the steroids and converts them to oximes. The oximes of the steroids formed have both *cis* and *trans* isomers, manifesting as single or double peaks on chromatograms after column separation. In our experiment, the MRM chromatograms of five compounds, including testosterone, DHEA, androsterone, pregnenolone, and pregnane-3,17-diol-20-one, showed single peak. The MRM transition chromatograms of other compounds showed double peaks. For those compounds, the sum of the integration areas of both peaks (quantification ions) was used for the quantification. Most MRM transitions were derived from the protonated oxime molecules $[M+H]^+$. However, for DHT and androstanedione, the ion cluster $[M+H+CH_3CN]^+$ was used as the precursor ion and the dissociated ion $[M+H]^+$ was used as product ion. The transitions are 347 > 306, and 360 > 319, respectively. Double charged ions and transitions were used for progesterone oxime and pregnane-3,20-dione oxime. The transitions are *m/z* 214 $[M+2H+2CH_3CN]^{++}$ > *m/z* 173 $[M+2H]^{++}$, and 215 $[M+2H+2CH_3CN]^{++}$ > *m/z* 174 $[M+2H]^{++}$, respectively. The transition ions and their possible fragmentation structures are listed in Table 1. The precursor to product ion transitions [quantification and target (confirmation) ions], dwell times, cone voltage, and collision energy for each standard and internal standard are listed in Table 2. The MRM method which contains all the MRM transitions is designed with 7 functions. Each function covers a different time window throughout the chromatographic run (see Table 2). The ion response ratio (quantification/confirmation) acceptability range was established as ±50% relative of the mean value of the product ion ratios obtained from the calibrators. Analytes were identified by comparing the retention times and the MRM transitions with those of the corresponding calibrators and QC samples. The MRM chromatograms of the quantification ions related to the hydroxylamine derivatives of the steroids (1 ng/mL) are listed in Fig. 1. Data were processed with the Targetlynx program (Waters), using peak area ratio for quantification.

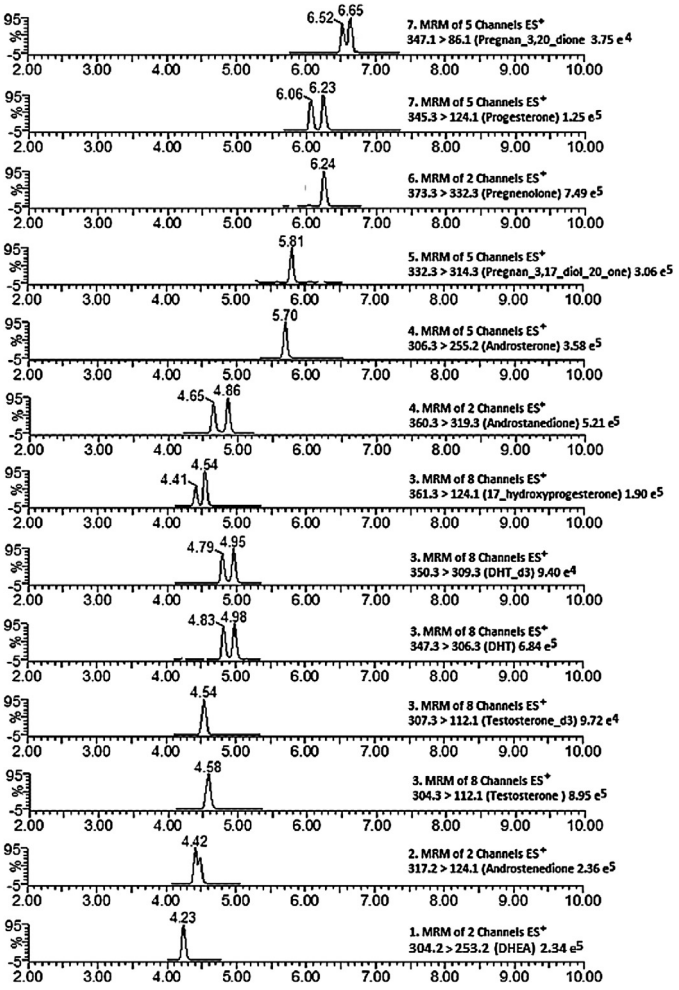


Fig. 1. Chromatograms of the quantification MRM transitions (hydroxylamine derivatives of the steroids).

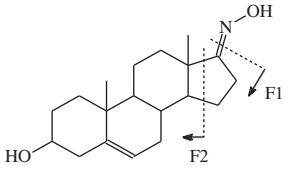
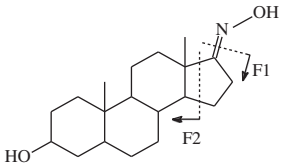
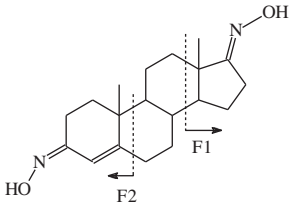
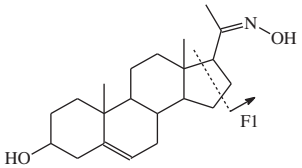
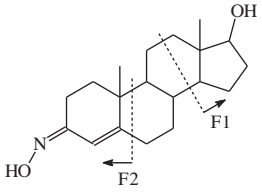
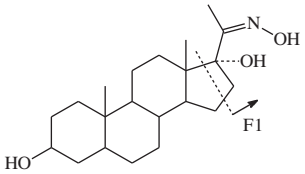
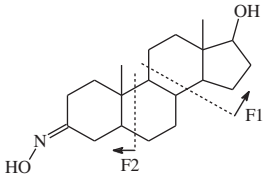
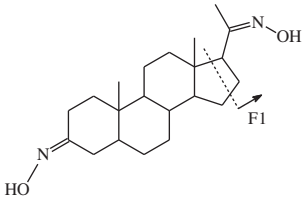
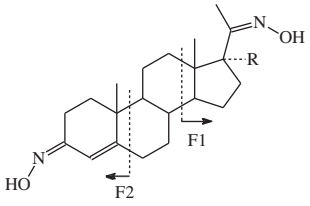
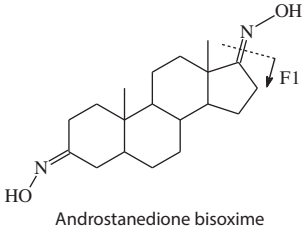
2.8. Preparation of standards and QC solutions

A working standard solution containing 10 steroids was prepared to form a final concentration of 1000 ng/mL for each compound. Charcoal stripped fetal bovine serum (CSS) was spiked with the working solution of steroids to achieve the following calibration standard concentrations: 10, 50, 100, 500, 1000, 2000, and 5000 pg/mL. Quality control (QC) samples, with the concentration of 30, 800, and 4000 pg/mL, were also prepared in the same way and were run in each assay. This standard curve was used for the analysis of the steroids in media. For the cell analysis, the standard curve points were same as those of the media, but the solution was prepared using ethyl acetate. Internal standard solution was prepared by combining all the internal standards together and accurately diluting the mixture with methanol to get the final concentration of 20 ng/mL for each compound. The deviation and the coefficient of variation (CV%) were used to evaluate the accuracy and precision of the method. Standard curve point variation was less than 15% (less than 20% for LLOQ) of the nominal values. The steroids in the QC samples were determined via the daily calibration curves and the accuracy was committed within 20% of the nominal values. The intervals of the intra-day and inter-day precision for QC samples were CV% within 20%. All of the stock solutions were stored at −20 °C. Standard curve calibrators were prepared for each day of analysis.

2.9. Preparation and extraction of the samples

The media samples were separated and concentrated using solid phase extraction (SPE) on Oasis HLB cartridges (Waters, Ontario, CA). All of the HLB cartridges (waters) were first activated and washed with 1 mL of methanol and then equilibrated with 1 mL of distilled water. Then, aliquots of 3 mL of media (containing 10 μL of internal standards in each sample) were loaded to the HLB cartridges. The cartridges were washed with 1 mL of distilled water, and after that, the analytes were eluted in 1 mL of acetone and evaporated to dryness using Centrivap. The residues were used for the analysis of steroids after derivatized by hydroxylamine hydrochloride. The cell samples were extracted with 1 mL of ethyl acetate for 30 min at room temperature and then centrifuged for 5 min at 20,000 rcf. The ethyl acetate solution of each sample was transferred into a 2 mL of Eppendorf tube and evaporated to dryness at 40 °C using Centrivap. The residue was used for the derivatization and analysis. The mouse serum samples were extracted by ethyl acetate using a similar procedure to that used for cell samples. Equivolumes (80 μL) of the serum were used from each sample for the extraction.

Table 1
The transition ions of the steroids oximes used for the analysis.

Structures*	Ions (*m*/*z*)*	Structures*	Ions (*m*/*z*)*
 DHEA oxime	304 $[M+H]^+$ 253 $[F1\text{-}H_2O\text{-}H]$ 213 $[F2\text{-}H_2O\text{-}H]$	 Androsterone oxime	306 $[M+H]^+$ 255 $[F1\text{-}H_2O\text{-}H]$ 215 $[F2\text{-}H_2O\text{-}H]$
 Androstenedione bisoxime	317 $[M+H]^+$ 112 [F1 + H] 124 [F2 + H]	 Pregnenolone oxime	332 $[M+H]^+$ 373 $[M+H+CH_3CN]^+$ 86 [F1 + H]
 Testosterone oxime	304 $[M+H]^+$ 112 [F1]; or $[F2+H\text{-}CH_2+2H]$ 124 [F2 + H]	 Pregnan-3,17-diol-20-one oxime	350 $[M+H]^+$ 332 $[M+H\text{-}H_2O]$ 314 $[M+H\text{-}2H_2O]$ 86 $[F1\text{-}H_2O+H]$
 DHT oxime	306 $[M+H]^+$ 347 $[M+H+CH_3CN]^+$ 107 $[F1\text{-}H_2O\text{-}H]$; or $[F2\text{-}CH_3\text{-}3H]]$ [F2-OH-2H]	 Pregnan-3,20-dione bisoxime	347 $[M+H]^+$ 215 $[M+2H+2CH_3CN]^{++}$ 174 $[M+2H]^{++}$ F1 + H]
 [1] R=H, Progesterone bisoxime [2] R=OH, 17-OH-progesterone bisoxime	[1] 345 $[M+H]^+$ 214$[M+2H+2CH_3CN]^{++}$ 173 $[M+2H]^{++}$ 124 [F2 + H]; or $[F1+H\text{-}CH_2\text{-}2H]$ [2] 361 $[M+H]^+$ 124 [F2 + H], or $[F1+H\text{-}CH_2\text{-}H_2O]$ 112 [F2 + H-CH + H]	 Androstanedione bisoxime	319 $[M+H]^+$ 360 $[M+H+CH_3CN]^+$ 286[F1-H] or [M + H-2OH + H]

* F1 and F2 indicate the possible positions of fragmentation to produce the transition ions.

2.10. Sample derivatization

Hydroxylamine hydrochloride (Sigma, ON, CA) was required for the derivatization reaction. The dried standards and sample residues were first reconstituted in 50 μL of methanol, vortex mixed for 1 min, and then 50 μL of 100 mmol of hydroxylamine hydrochloride solution was added into each standard and sample, respectively. All of the samples and standards were vortexed, sealed, and then heated at 60 °C for 1 h. After completing the oxime formation, the samples were vortexed for 10 s and centrifuged at 20,000 rcf for 3 min. The supernatants were transferred to vials (with insert) and 10 μL injected into the UPLC–MS/MS system for analysis.

2.11. PTEN knock-out in vivo model of prostate cancer

The prostate-specific conditional PTEN knockout ($PTEN^{-/-}$) mouse tumor model was generated in house [16] by crossing the ARR2probasin-Cre transgenic line, a gift from Dr. P. Roy-Burman (University of Southern California), with $PTEN^{flox/flox}$ mice from Dr. Tak Mak (University of Toronto). Genomic DNA was isolated from tail clips of F2 offspring and was analyzed by PCR using $PTEN^{flox}$ and Cre-specific primers to confirm PTEN deletion in the offspring mice. Correspondingly, the conditional PTEN knockout mouse model used in these experiments is androgen dependant [17]. Mice were housed in polycarbonate cages with corn-cob bedding at a temperature of 20–23 °C and had a 12-h photoperiod.

Table 2
MRM transitions of steroids (hydroxylamine derivatives).

compound	Transition	Cone volt.	Col. energy	Dwell	Time window
DHEA	304.30 > 213.2 304.30 > 253.2*	40 40	21 18	0.05	4.0–4.9
Androstenedione	317.30 > 112.1 317.30 > 124.1*	35 35	25 25	0.05	4.2–5.2
Testosterone	304.30 > 112.1* 304.30 > 124.1	50 50	30 30	0.05	4.2–5.6
DHT	306.30 > 107.3 347.30 > 306.3*	50 50	28 12	0.05	4.2–5.6
Androstanedione	360.30 > 319.30* 319.30 > 286.30	40 40	12 20	0.05	4.2–5.3
17-hydroxyprogesterone	361.30 > 112.1 361.30 > 124.1*	50 50	36 35	0.05	4.2–5.6
Androsterone	306.30 > 215.0 306.30 > 255.2*	40 40	32 20	0.05	5.5–6.6
Pregnan-3,17-diol-20-one	332.30 > 314.3* 350.10 > 86.1	35 35	20 30	0.05	5.5–6.6
Pregnenolone	332.30 > 86.1 373.31 > 332.3*	40 40	32 12	0.08	5.8–6.9
Progesterone	214.20 > 173.2 345.30 > 124.1*	30 45	8 30	0.05	5.8–7.4
Pregnan-3,20-dione	215.20 > 174.2 347.10 > 86.0*	30 30	10 30	0.05	5.8–7.4
Testosterone_d3	307.30 > 112.1* 307.3 > 124.1	50 50	30 30	0.05	4.2–5.6
DHT_d3	309.30 > 110.3 350.30 > 309.3*	50 50	28 12	0.05	4.2–5.6

* Quantification ion.

Mice were given commercial mouse diet and water *ad libitum*. Mice were cared for and treated in accordance with the principles and guidelines of the Canadian Council on Animal Care.

Male PTEN$^{-/-}$ mice were treated with 1% sucrose (controls, $n = 7$) or 0.17 g/L of pomegranate fruit extract in 1% sucrose (samples, $n = 7$). Following genotyping analysis mice were recruited at the age of 6 weeks and were allowed to consume either 1% sucrose or pomegranate fruit extract containing water *ad libitum*. Upon reaching 20 weeks of age, mice were euthanized using CO_2 asphyxiation and the blood was collected from each mouse by cardiac puncture. Blood samples were spun in a tabletop centrifuge at 5000 rpm for 5 min at 4 °C, and the sera were collected for steroid analyses.

2.12. Western blot and total prostate specific antigen (tPSA) analysis

Both LNCaP and 22RV1 cells were cultured in RPMI 1640 culture media containing 5% CSS with and without POM (12 μg/mL) as described above. After 48 h, the cells were harvested using a plastic scraper and transferred into Eppendorf micro centrifuge tubes. For Western blots, cell lysate buffer (consisting of 50 mM Tris–Cl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 20 mM NaF, 1 mM Na_3VO_4, 0.5% NP-40, 1% Triton X-100, 1 mM PMSF, 10 μg/mL Apoprotein, and 10 μg/mL leupeptin) was added to cell pellets and vortexed. The suspension was incubated for 30 min on ice. The samples were centrifuged at 14000 rpm for 15 min and the supernatant was collected. Protein concentration was quantified using Bio-rad BCA protein Assay. Thirty microgram of protein were loaded into 12% SDS-acrylamide gels. Proteins were transferred to nitrocellulose membrane. The membrane was blocked using Odyssey Buffer for 2 h and probed with primary antibody at 4 °C over night followed by room temperature for 3 h. Secondary antibody was probed for 1 h and scanned using Odyssey. The following antibodies were used: α-β-actin (loading control, Sigma-Aldrich), α-AKR1C1, α-AKR1C3, α-SRD5A2 (Abcam), α-CYP11A1 (Santa Cruz), α-CYP17A1 (Donated by Dr. Hales of Illinois University), α-CYB5A, α-HSD3B2, α-HSD17B3 (Abnova), α-HSD17B1, α-SRD5A1 (Novus Biologicals), α-Androgen Receptor (AR, Santa Cruz), and α-Prostate specific antigen (PSA, Santa Cruz)

Cell culture media from controls and samples treated with POM at the four different concentrations (2, 5, 8, 12 μg/mL) were collected for the PSA analysis. The tPSA concentrations were analyzed using Cobas 411 analyzer (Roche). Reagents and working solutions kit were provided by Roche. The analyzer automatically calculated the concentration of each sample (in ng/mL). Results are determined by a 2-point calibration curve generated by the instrument and a master curve provided via the reagent barcode. Calibration range was 0.002–100 ng/mL and samples were diluted and repeated if the tested values were over 100 ng/mL.

2.13. Statistical analysis

All of the data obtained from the experiments were analyzed using Excel *t* test. The values of controls and POM treated samples were compared and the results were expressed as mean ± SDs in the figures. Significance was considered at $P < 0.05$.

3. Results

3.1. Components of pomegranate extract

Several chemical constituents of the water soluble extract, including gallic acid, ellagic acid, caffeic acid, luteolin, hexahydroxydiphenic acid, cyanidin, and gallagyldilactone were separated and identified using preparative thin layer chromatography (TLC), HPLC (PDA), and LC/MS methods including MS scan and MRM, and compared with the authentic standards. Consideration should be made to the fact that POM contains an abundance of biologically-active constituents, for example, ellagitanins, punicaligins, gallotanins, anthocyandins, anthocyanins, flavonoids and their glycosides etc. [8,18,19], for which analysis was not completed. The PDA and TIC chromatograms of the water soluble extract are shown in Fig. 2.

3.2. Cell viability test

Theoretically, MTS reacts with live cells and is converted into a color formazan product that is soluble in tissue culture medium. This reaction is presumably accomplished by NADPH or NADH produced by dehydrogenases in metabolically active cells. However, in our experiment, we found that POM also reacted with MTS to produce a color which is similar with that of the formazan. As low as 0.4 μg/mL of POM could effectively cause a visual color change after mixed with MTS. This color change affected the detection of the UV absorbance of the formazan converted by live cells, and so any inhibition of cell viability by POM could not be accurately determined.

Trypan blue is one of the several stains recommended for use in dye exclusion procedures for viable cell counting. It is based on the principle that live cells do not take up Trypan blue, whereas dead cells do. Ten concentrations (0, 4, 8, 12, 16, 20, 100, 250, 500, 1000 μg/mL) of pomegranate extract were used for this study. The results indicated that POM started to inhibit cell growth at a concentration of 20 μg/mL in both cell lines: LNCaP and 22RV1. Determined IC_{50}s for LNCaP and 22RV1 were around 68 and 71 μg/mL, respectively, as shown in Fig. 3. These experiments were critical in that they provided a concentration range for us to use for steroidogenesis assays at which we were assured that we were working with living cells.

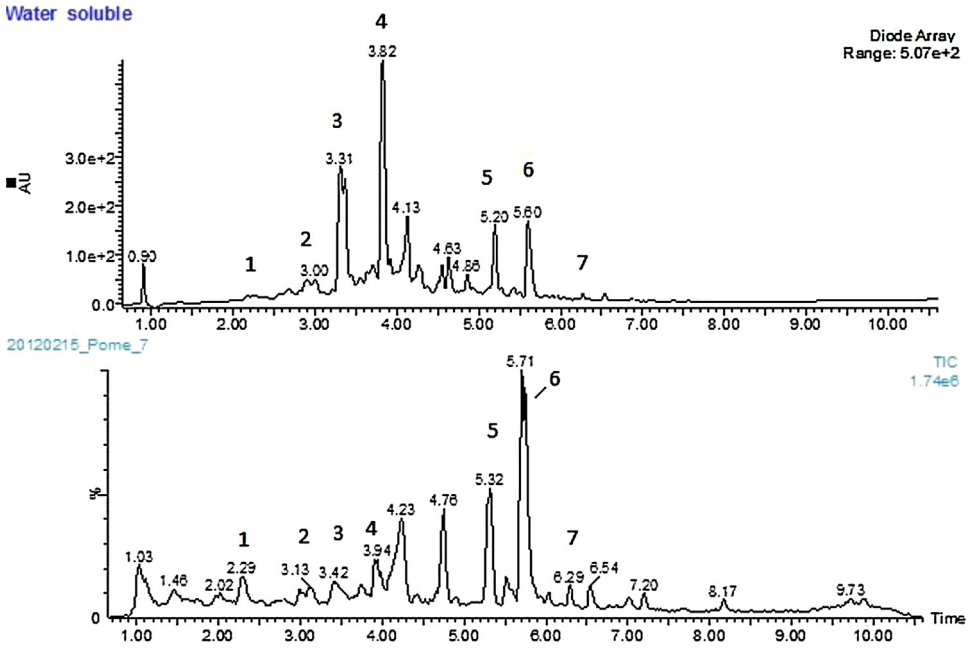


Fig. 2. PDA and TIC Chromatograms of Pomegranate water soluble extract.
((1) Gallic acid, (2) caffeic acid, (3) hexahydroxydiphenic acid, (4) gallagyldilactone, (5) cyanidin, (6) ellagic acid, (7) luteolin.)

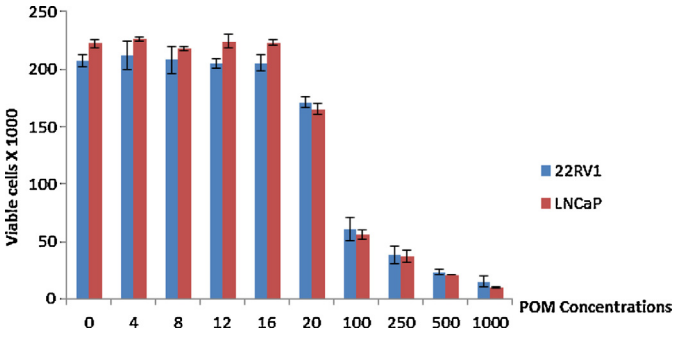


Fig. 3. Impact of the pomegranate extracts on cell viability (expressed as mean ± SD).

3.3. Progesterone assays for steroid biosynthesis

The 22RV1 cell line was used to study the activity of POM on steroid biosynthesis from progesterone precursor.

3.3.1. Culture media steroid data

The culture media was supplemented with 2 μg/mL of progesterone. All of the ten steroids monitored were detected in the media (Table 3). The concentrations (pg/3 mL) of nine steroids were quantified in the control media. They were: DHEA 23, androstenedione 122, 17-hydroxyprogesterone 400, testosterone 19, DHT 13, androsterone 12, pregnane-3,17-diol-20-one 8792; pregnenolone 90805, progesterone 629659, respectively (Table 3). Pregnane-3,17-dione was also found in the media, however, its MRM transitions showed interference with another unidentified compound and the concentration could not be quantified precisely. In comparison with the control, the concentrations of the detected compounds in the media of cells treated with POM dropped. The reduction ratios for androstenedione, 17-hydroxyprogesterone, pregnane-3,17-diol-20-one, pregnenolone, and testosterone were 36.1%, 29.2%, 18.3%, 48.0%, and 31.6%, respectively ($P < 0.05$). DHEA and androsterone were also decreased by POM in the media, but lack of significance. The concentration of the DHT in the treatment media was below the limit of the detection (LOD) of the method and also decreased compared with the value of the control (13 pg/3 mL).

3.3.2. Cell steroid data

In cells, DHEA, pregnane 3,17-diol-20-one, pregnenolone, and progesterone were detected and quantified (Table 3) and their concentrations were significantly decreased in the samples treated with POM. The ratios by which amounts of steroids decreased were 45.6%, 52.1%, 58.7%, and 24.8%, respectively ($P < 0.04$, Table 3). The LC/MS/MS data of other steroids including androstenedione, hydroxyprogesterone, testosterone, DHT, androsterone were not used for the calculation and evaluation because they were below the limit of quantification of the method.

3.4. DHEA assays for steroid biosynthesis

In the media of the sample set described above, which were spiked with progesterone, the concentrations of several steroids were still very low and therefore hardly detected. Moreover, progesterone did not enhance the levels of testosterone and DHT. For further evaluation and confirmation of the activity of POM, we designed a new group of experiments in which all of the culture media were supplemented with 2 μg/mL of DHEA. Two cells lines, 22RV1 and LNCaP, were used for the study. The results proved that, using DHEA, the concentrations of testosterone and DHT were significantly increased (see Tables 4 and 5) compared to experiments which used progesterone as an androgen precursor. The ability to determine higher concentrations increases the accuracy of the analysis.

Seven steroids including DHEA, androstenedione, testosterone, DHT, androstanedione, androsterone, and pregnenolone were identified and quantified in both media and cells of 22RV1. The concentrations of all of the seven steroids were significantly reduced in the POM treated samples ($P < 0.03$, Table 4). In cells, POM started inhibiting the biosynthesis of several steroids at 2 μg/mL. The inhibiting ratios (at 12 μg/mL) by which amounts of steroids decreased were 43.3%, 52.6%, 61.6%, 54.1%, 71.6%, 71.6%, 68.1%

Table 3
Steroids concentrations (mean ± SD) in the control and POM treated 22RV1 cells and media (n = 4, progesterone pre-cursor).

Compounds	In cells (pg/g)		Decreased ratios (%)[#]	In media (pg/3 mL)		Decreased ratios[#]
	Control	POM samples		Control	POM samples	
DHEA	627 ± 109	341 ± 24[*]	45.6	23 ± 2	19 ± 3	
Andostenedione	ND	ND		122 ± 17	78 ± 13[*]	36.1%
OH-progesterone	ND	ND		400 ± 43	283 ± 6[*]	29.2%
Testosterone	ND	ND		19 ± 2	13 ± 1[*]	31.6%
DHT	ND	ND		13 ± 3	ND[*]	>75%
Androsterone	ND	ND		12 ± 1	9 ± 2	
Pregnane-3,17-diol-20-one	7831 ± 53	3750 ± 80[*]	52.1	8792 ± 339	7186 ± 246[*]	18.3%
Pregnenolone	926,078 ± 99268	382,074 ± 2716[*]	58.7	90,805 ± 3591	47,251 ± 1435[*]	48.0%
Progesterone	835,022 ± 36620	628,146 ± 3741[*]	24.8	629,659 ± 21,080	676,645 ± 45,980	

ND: not detected or below the limit of the detection.
[*] P < 0.05 denoting significance.
[#] Decreased ratios indicated the reduced percentages between samples and controls, which are calculated using formula: [1 − (sample value/control value)] × 100%. Only the ratios denoting significances are listed in the table.

for DHEA, androstenedione, testosterone, DHT, androstanedione, androsterone, and pregnenolone, respectively.

In the media samples of cell line 22RV1, the concentration of androstendione and pregnenolone were found to be decreased by 2 μg/mL of POM in comparison with that of the control (Table 4). Testosterone, DHT, and androsterone were not affected at this concentration. When the concentration of POM increased to 5 μg/mL in the media, all of the steroids listed above dropped. Androstendione, testosterone, DHT, androstanedione, androsterone, and pregnenolone levels were significantly reduced when the cells were treated with 12 μg/mL of POM (P values: 0.004, <0.001, <0.001, 0.004, 0.025, and 0.018, respectively). The ratios by which amounts of steroids decreased were 56.3%, 60.0%, 51.0%, 55.8%, 57.3%, and 68.0%, respectively.

The steroids in the LNCaP cells and media were also evaluated and quantified, and seven compounds were clearly detected in the samples prepared from both the media and the cells. POM were found to reduce the levels of five steroids: androstenedione, testosterone, DHT, androstanedione, and androsterone at 5 μg/mL (P < 0.05, Table 5), which were confirmed in samples from both cells and media from the LNCaP cell line. When treated with 12 μg/mL of POM, the concentrations of DHEA, androstenedione, testosterone, DHT, androstanedione, androsterone, and prognenolone in cells were reduced by 32.8%, 29.4%, 25.5%, 45.1%, 52.6%, 38.5%, and 31.4%, respectively (P values: 0.006, 0.001, 0.002, 0.01, 0.002, 0.002, and 0.037, respectively). In media, the ratios by which amounts of steroids decreased were 47.9%, 41.9%, 41.3%, 41.0%, 50.7%, and 47.5.0% for androstenedione, testosterone, DHT, androstanedione, androsterone, and pregnenolone, respectively (P < 0.02).

3.5. Serum steroids determined for PTEN ko mice fed POM

Blood was collected from POM and control treated mice upon euthanisation at 20 weeks of age. An 80 μL aliquot of serum was extracted using ethyl acetate, and the ethyl acetate extraction was dried, derivatized using hydroxylamine, and then analyzed using UPLC/MS/MS. DHEA, testosterone, pregnenolone, and progesterone were identified and quantified in controls and samples respectively (Table 6). When compared with the controls, the average values of the three steroids: DHEA, testosterone, and pregnenolone were found to be reduced by the treatment of mice with POM. The ratios by which amounts of steroids decreased were 42.1%, 80.3%, and 36.5%, respectively (P values: 0.006, 0.02, and 0.023, respectively). A decrease in prostate weight was also determined for POM treated animals in accordance with this drop in serum steroids (data not shown).

3.6. Western blot and tPSA analysis

Western blotting was used to quantitate and compare protein levels of enzymes in the steroid biosynthesis pathway as well as AR and its downstream sentinel of activation, PSA. The results showed that the levels (band intensity) of PSA was significantly reduced (P = 0.004, Fig. 4) and AKR1C3 (P < 0.001) and AR (P = 0.025) were

Table 4
Concentrations of the steroids (mean ± SD) in the cells and media of 22RV1 (n = 4, DHEA pre-cursor, cells unit ng/g, media unit ng/3 mL).

	DHEA	Androstenedione	Testosterone	DHT	Androstanedione	Androsterone	Pregnenolone
Cells samples							
Cell_control	1657 ± 17	3.10 ± 0.01	1.38 ± 0.06	0.61 ± 0.01	1.48 ± 0.25	2.68 ± 0.12	13.54 ± 0.98
Cell_POM_2 μg	1051 ± 182	2.27 ± 0.35	0.84 ± 0.17	0.28 ± 0.06	0.62 ± 0.11	1.11 ± 0.18	13.11 ± 1.34
Cell_POM_5 μg	1182 ± 127	1.98 ± 0.12	0.90 ± 0.04	0.32 ± 0.01	0.58 ± 0.02	0.83 ± 0.15	9.07 ± 0.08
Cell_POM_8 μg	829 ± 103[*]	1.37 ± 0.16[*]	1.17 ± 0.15	0.45 ± 0.02	0.48 ± 0.02[*]	1.67 ± 0.21	4.53 ± 0.03[*]
Cell_POM_12 μg	939 ± 124[*]	1.47 ± 0.09[*]	0.53 ± 0.05[*]	0.28 ± 0.02[*]	0.42 ± 0.01[*]	0.76 ± 0.06[*]	4.32 ± 0.20[*]
Decreased ratios[#]							
Cell_POM_8 μg	50.0%	55.8%			67.5%		66.6%
Cell_POM_12 μg	43.3%	52.6%	61.6%	54.1%	71.6%	71.6%	68.1%
Media samples							
Media_Control	413 ± 26	8.09 ± 1.00	0.75 ± 0.03	0.10 ± 0.002	1.02 ± 0.02	1.24 ± 0.24	0.25 ± 0.06
Media_POM_2 μg	375 ± 180	6.14 ± 0.88	0.95 ± 0.03	0.12 ± 0.01	1.38 ± 0.03	1.42 ± 0.04	0.21 ± 0.01
Media_POM_5 μg	402 ± 26	7.02 ± 0.31	0.71 ± 0.03	0.093 ± 0.005	1.21 ± 0.04	1.21 ± 0.11	0.14 ± 0.02
Media_POM_8 μg	344 ± 10	5.10 ± 0.10	0.52 ± 0.06[*]	0.074 ± 0.005[*]	0.71 ± 0.01[*]	0.58 ± 0.02[*]	0.11 ± 0.01[*]
Media_POM_12 μg	310 ± 5	3.54 ± 0.54[*]	0.30 ± 0.02[*]	0.049 ± 0.005[*]	0.45 ± 0.01[*]	0.53 ± 0.03[*]	0.08 ± 0.01[*]
Decreased ratios[#]							
Media_POM_8 μg			30.6%	26.0%	30.4%	53.2%	56.0%
Media_POM_12 μg		56.3%	60.0%	51.0%	55.8%	57.3%	68.0%

[*] P < 0.05 denoting significance.
[#] Decreased ratios indicated the reduced percentages between samples and controls, which are calculated using formula: [1 − (sample value/control value)] × 100%. Only the ratios denoting significances are listed in the table.

Table 5
Concentrations of the steroids (mean ± SD) in the cells and media of LNCaP (n = 4, DHEA pre-cursor, cells unit ng/g, media unit ng/3 mL).

	DHEA	Androstenedione	Testosterone	DHT	Androstanedione	Androsterone	Pregnenolone
Cell samples							
Cell_control	2137 ± 140	14.45 ± 1.90	8.49 ± 0.04	5.21 ± 0.28	15.90 ± 1.99	11.88 ± 0.28	16.01 ± 3.02
Cell_POM_2 μg	2527 ± 160	16.01 ± 0.96	8.36 ± 0.10	4.56 ± 0.04	16.50 ± 0.30	12.14 ± 0.26	19.18 ± 3.44
Cell_POM_5 μg	1974 ± 54	13.11 ± 1.71	6.72 ± 0.54*	3.45 ± 0.25*	12.11 ± 0.45*	7.61 ± 0.80*	17.83 ± 3.32
Cell_POM_8 μg	1610 ± 92	8.76 ± 0.08*	5.99 ± 0.09*	3.86 ± 0.19*	10.71 ± 1.73*	9.49 ± 1.40*	10.04 ± 0.36*
Cell_POM_12 μg	1437 ± 155*	10.21 ± 1.02*	6.33 ± 0.35*	2.86 ± 0.38*	7.54 ± 0.11*	7.30 ± 1.00*	10.98 ± 1.23*
Decreased ratios#							
Cell_POM_5 μg			21.0%	33.8%	23.8%	35.9%	
Cell_POM_8 μg		39.4%	29.4%	25.9%	32.6%	20.1%	37.3%
Cell_POM_12 μg	32.8%	29.4%	25.5%	45.1%	52.6%	38.5%	31.4%
Media samples							
Media_Control	494 ± 696	14.21 ± 2.46	4.34 ± 0.80	1.21 ± 0.17	9.63 ± 0.41	4.42 ± 0.64	0.12 ± 0.01
Media_POM_2 μg	441 ± 11	16.23 ± 0.94	4.56 ± 0.31	1.14 ± 0.65	9.55 ± 0.10	3.49 ± 0.46	0.089 ± 0.01
Media_POM_5 μg	640 ± 28	12.70 ± 0.63	3.46 ± 0.27	1.02 ± 0.10	7.62 ± 0.38	3.18 ± 0.07	0.13 ± 0.01
Media_POM_8 μg	438 ± 52	9.64 ± 0.19*	2.27 ± 0.14*	0.61 ± 0.05*	5.89 ± 0.08*	1.88 ± 0.07*	0.065 ± 0.003*
Media_POM_12 μg	424 ± 11	7.41 ± 0.27*	2.52 ± 0.08*	0.71 ± 0.07*	5.68 ± 0.09*	2.18 ± 0.03*	0.063 ± 0.01*
Decreased ratios#							
Media_POM_8 μg		32.2%	47.7%	49.6%	38.8%	57.5%	45.8%
Media_POM_12 μg		47.9%	41.9%	41.3%	41.0%	50.7%	47.5%

* P < 0.05 denoting significance.
\# Decreased ratios indicated the reduced percentages between samples and controls, which are calculated using formula: [1 − (sample value/Control value)] × 100%. Only the ratios denoting significances are listed in the table.

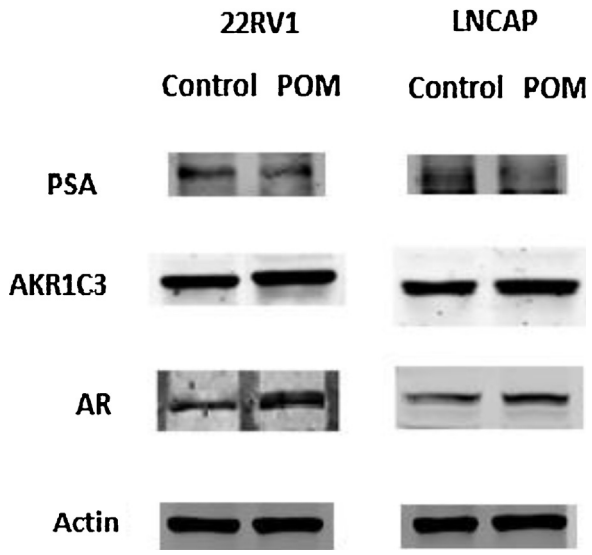


Fig. 4. Western blot analysis on the enzymes expressed on the steroids biosynthesis.

significantly enhanced in the POM treated LNCaP cells. The same results were also observed in the POM treated 22RV1 samples. The P values were 0.018, 0.029, and 0.008 for PSA, AKR1C3 and AR, respectively. No statistic significances were found for other enzymes tested in this experiment.

Overall, total PSA concentrations were decreased in the samples treated by POM compared with controls in both cell lines. The tPSA concentrations in the LNCaP cells treated with vehicle, 2, 5, and 12 μg/mL of POM were 329.2, 308.3, 239.4, and 261.9 ng/mL, respectively. In contrast, concentrations of tPSA in the 22RV1 cell line were significantly lower with values of 1.26, 0.91, 0.93, 0.94, and 0.81 ng/mL for control and samples treated with 2, 5, 8, 12 μg/mL POM, respectively.

4. Discussion

CRPC remains a major cause of PCa mortality and a challenge for physicians to treat medically. Residual androgens have been determined in tumor tissues and are at levels sufficient to activate AR which could, in part, drive this process.

Table 6
The concentrations of steroids (mean ± SD, ng/mL) in the serum of the mice of conditional PTEN Knock out prostate cancer model (n = 7).

Compounds	Control	POM	Decreased ratios (%)#
DHEA	13.17 ± 2.94	7.63 ± 2.50*	42.1
Testosterone	91.39 ± 65.15	17.96 ± 9.30*	80.3
Pregnenolone	285.91 ± 60.81	181 ± 53*	36.5
Progesterone	3.20 ± 1.98	5.91 ± 8.37	

* P < 0.05 denoting significance.
\# Decreased ratios indicated the reduced percentages between samples and controls, which are calculated using formula: [1 − (sample value/control value)] × 100%. Only the ratios denoting significances are listed in the table.

Testosterone and dihyrotestosterone (DHT) are the major androgens produced through three biosynthesis pathways: Δ^4, Δ^5, and backdoor pathways (Fig. 5) [4,20–24]. The proliferation and survival of the prostate cancer cells are dependent on androgen mediated AR signaling and gene expression. The present study examines the effects of pomegranate extracts (POM) on steroid biosynthesis *in vitro* using PCa cell lines (22RV1 and LNCaP) alongside an *in vivo* model representing PCa progression (transgenic mouse conditional PTEN knockout prostate cancer model). Since most of the steroids (testosterone and DHT in particular) are present in very low concentrations in cells and media, DHEA and progesterone were used as precursor substrates to help boost cell production of steroids and bring their respective levels up from the detection threshold for the analysis. The results showed that compared to controls POM decreases the production of testosterone, DHT, DHEA, androstenedione, androstanedione, androsterone, and pregnenolone generated through steroid biosynthesis pathways. The testosterone and DHT concentrations were significantly decreased in samples treated with POM, which provides mechanistic rationale for the observations reported clinically for the effects of POM in the treatment of prostate cancer patients [11–13].

Supplementing with 2 μg of DHEA in cell culture significantly increased the concentration of androstenedione, androstanedione, androsterone, testosterone, and DHT compared with the samples which were not spiked (data not provided), confirming the steroid biosynthesis scheme shown in Fig. 5: DHEA → androstenedione → androstanedione → androsterone (or DHT), and DHEA → androstenedione or *5-androstenediol* (not tested in this method) → testosterone → DHT (Fig. 5) [20,25]. After samples were treated with POM, the concentrations of above six

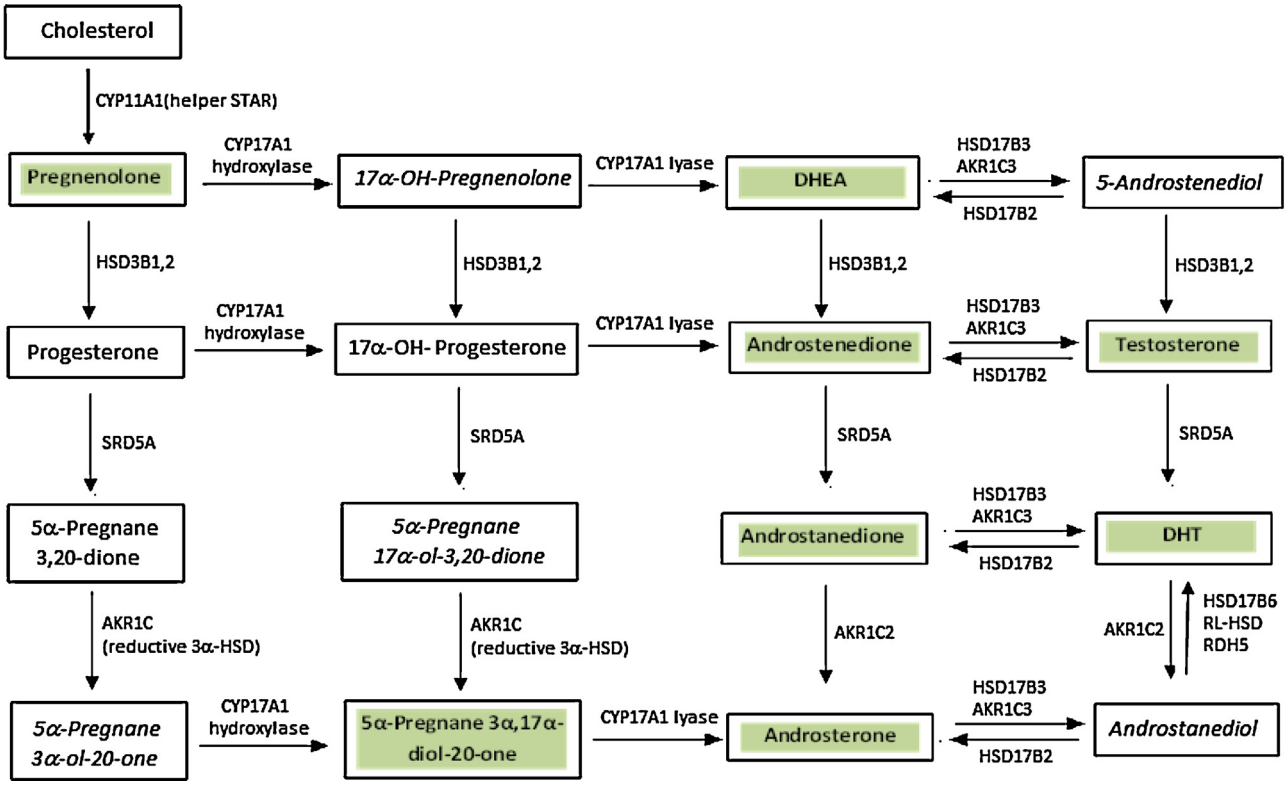


Fig. 5. The classical (Δ^4 and Δ^5) and back door pathways of androgen biosynthesis. POM impact on the production of multiple steroids. Shading denotes a significant reduction and *italics* denote steroids not analysed.

compounds were significantly reduced in both cells and media of both LNCaP and 22RV1 cell lines.

In the progesterone treated 22RV1 cells, DHEA and pregnane-3,17-diol-20-one were found in both cell and media samples. Androstenedione, hydroxyprogesterone, testosterone, and DHT were identified from the media, but they were below of the detection limit in the cells. The concentrations of pregnane-3, 17–diol-20-one in the samples treated by POM were significantly reduced compared with controls. Upon adding progesterone in the media, pregnane-3, 17–diol-20-one was increased to very high levels in the samples derived from both the cells and media. However, 17-hydroxyprogesterone, which was expected to produce high values in cells using this method, was below the detection limit. Moreover, its concentration in media was 20 fold lower than pregnane-3, 17–diol-20-one. This result suggests that pregane-3, 17–diol-20-one may come from the backdoor pathway: progesterone → pregnane-3,20-dione → pregnane-3-ol-20-one (not tested) → pregnane -3,17-diol-20-one. In the DHEA treated group, the concentrations of androstenedione, androstanedione, and androsterone in cell samples were consistently higher than testosterone and DHT, as confirmed in both LNCaP and 22RV1 cell lines. The above results suggest that, in prostate cancer cells, steroid biosynthesis might favor the backdoor pathway over the classical Δ^4 and Δ^5 pathways, which supports the hypothesis described by Mostaghel [20]. After spiking progesterone into cells in culture, the concentrations of testosterone and DHT in media were still low (below 20 pg/mL). The low concentrations implied difficulty of detection and thus may cause the result analysis to be misleading. However, upon supplementing the culture media with DHEA, the concentrations of testosterone and DHT were boosted up to nanogram levels, minimizing potential analytical error and sample variation. We found that spiking DHEA into the culture media was a more effective way of enhancing testosterone and DHT production than by using progesterone. The resulting *in vitro* system is very effective for studying the biosynthesis of testosterone and DHT. It may also be very useful to consider this assay in the development and evaluation of drugs used as steroidogenesis inhibitors, especially, when the concentrations of the steroids are below the detection limit for analysis by LC/MS/MS. In summary, steroid inhibition profiles for cells and the culture media corresponded very well. Using cells for steroid analysis, it is very important to have sufficient quantities and accurately weigh the cells. The advantages of using media for the steroid analysis are that media provides enough volume for extraction and the accuracy and variation of results determined for samples are not related with or restricted by the weight, as it could be in the case of cells.

The activity of steroid biosynthesis enzymes may be affected by any of the following: (1) modulation enzyme levels, (2) substrate concentration, and (3) the presence of enzyme inhibitors or activators [26]. From our Western blot results, we can see that POM impacts the expression of several proteins including PSA, AKR1C3 and AR. AR activity would theoretically be initially suppressed by POM treatment and lead to the down-regulated expression of PSA. Our Western blot results do indeed confirm that PSA levels in both LNCaP and 22RV1 cells were reduced upon POM treatment. However, in our experiment, the concentration of AR and AKR1C3 were found to be enhanced. We attempt to rationalise this observation as follows: POM treatment reduces testosterone and DHT, which consequently activates a negative feedback process to selectively induce intratumoral expression of AKR1C3 and AR, similar to the effects of steroidogenic inhibitors as previously reported [27,28].

These data also therefore further support the suggestion that POM impacts steroid biosynthesis, as we have demonstrated here using UPLC/MS/MS. However, steroid biosynthesis is not only dependent on the expression of the biosynthesis enzymes, but also the concentration of inhibitors and substrates. Enzyme inhibitors alter the catalytic action of enzymes and consequently slow down, or in some cases, stop catalysis. There are three types of enzyme

inhibition: competitive, non-competitive and substrate inhibition [29–31]. POM contains a wealth of active chemical compounds such as flavonoids, tannins, and anthocyanidins which could potentially compete with the steroids for the active sites of the enzymes (binding), or alter the structures of the enzymes in the way that they cannot mediate conversion of the substrates (steroids), and thus slow down or halt activity of the enzymes.

The expression of PSA is mainly induced by androgens under the regulation of the AR at the transcriptional level. Activation of PSA transcription involves many different proteins that are recruited directly and indirectly by the AR. Castrate resistant prostate cancers largely depend on AR signaling for growth and continue to express high levels of AR and AR-regulated genes, such as PSA [32]. The results from our steroid profiling analysis indicate that POM impact steroid biosynthesis pathways and reduce the concentration of testosterone and DHT in both LNCaP and 22RV1 cell lines. At reduced steroid concentrations, AR activation would be suppressed leading to down-regulated expression of PSA. The active compounds in POM may also compete with the steroids for the active binding sites on AR and further impact AR signaling and regulation. Our Western blotting results indicate that PSA levels in both 22RV1 and LNCaP cell lines are reduced when POM was dosed into culture at 12 μg/mL and higher. tPSA values in the media samples, as determined using the Cobas analyzer, also confirmed that the POM treated samples were lower than controls. Interestingly, our *in vivo* data supports this observation. The PTEN Knockout model mouse represents a comprehensive model for tumor initiation and progression through all stages of prostate cancer to metastatic disease. POM was administered orally to mice and was found to be non-toxic when dosed chronically in drinking water at a concentration of 0.17 g/L. The results of the serum analysis show that the concentrations of DHEA, testosterone, and pregnenolone were significantly decreased upon treatment with POM. The ratios by which amounts of steroids decreased were 42.1%, 80.3%, and 36.5%, respectively (Table 6). This result was equivalent to our *in vitro* data generated using the LNCaP and 22RV1 cell lines. We are able to conclude therefore that POM impact the androgen biosynthesis pathways *in vitro* and that this phenomenon manifests *in vivo* in the PTEN ko model for prostate cancer. Our results therefore provide evidence to support a mechanistic rationale for the observed efficacy of pomegranate supplementation in CRPC patients [12,13].

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