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Pharmacological upregulation of prostate-specific membrane antigen (PSMA) expression in prostate cancer cells

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Background: Prostate-specific membrane antigen (PSMA)-based imaging and therapy are increasingly used for prostate cancer management. However, limitations are a low PSMA expression in certain patients. Androgen receptor axis inhibition can induce PSMA expression in vitro. We hypothesized that different approved compounds upregulate PSMA expression and tested their effect in vitro.

Methods: Androgen receptor (AR) expressing prostate cancer (LNCaP) and epithelial prostate cells (PNT1A) were treated for 7 days with enzalutamide, dutasteride, rapamycin, metformin, lovastatin, and acetylsalicylic acid (ASA). PSMA and AR protein expression was assessed using flow cytometry, immunocytochemistry and immunoblotting. Furthermore, uptake and internalization of ^{177}Lu-PSMA-617 was performed.

Results: Enzalutamide and dutasteride led to a significant (both $P < 0.05$) upregulation of PSMA surface levels in LNCaP cells. In addition, treatment with rapamycin showed a non-significant trend toward PSMA upregulation. No changes were detected after treatment with vehicle, metformin, lovastatin, and ASA. Total PSMA protein expression was significantly enhanced after treatment with enzalutamide and rapamycin (both $P < 0.05$), whereas dutasteride led to a non-significant upregulation. Uptake of ^{177}Lu-PSMA-617 was significantly increased after treatment of LNCaP with enzalutamide, dutasteride, and rapamycin ($P < 0.05$). In addition, internalization was significantly increased by enzalutamide and rapamycin ($P < 0.05$), and non-significantly increased by dutasteride.

Conclusion: In conclusion, our data provide new insights into the effect of different approved pharmacological compounds that can markedly upregulate PSMA expression and radioligand uptake in vitro. Pharmacologically induced PSMA expression may prove useful to improve prostate cancer detection and to enhance anticancer effects in PSMA-based therapy.

KEYWORDS
androgen antagonist, androgen receptor, ^{177}Lu-PSMA-617, prostate cancer, prostate-specific membrane antigen

1 | INTRODUCTION

Prostate-specific membrane antigen (PSMA) is a type II transmembrane protein, which has been first described and cloned in 1993.[1] Histological samples show a gradually increasing PSMA expression from benign prostatic hyperplasia to adenocarcinoma of the prostate.[2,3] A high PSMA expression is mainly observed in high-grade and metastatic prostate cancer, making PSMA a promising target for

imaging and therapy.[4] Positron emission tomography (PET) using PSMA ligands such as ^{68}Ga-PSMA-11 has markedly enhanced the sensitivity of prostate cancer imaging.[5] Especially the detection of metastases in patients with biochemical recurrence has been improved compared to other morphologic and molecular imaging modalities.[6] In addition, improved diagnostic accuracy in primary prostate cancer and the feasibility of multimodal image-guided prostate fusion biopsy has been demonstrated.[7,8] However, despite these improvements, the detection rate is still limited in certain patients predominantly presenting with low volume disease at the time of recurrence and in patients with low-grade cancer at diagnosis.[9] Short-term pharmacological boosting of PSMA expression might be a valuable concept to improve cancer detection using PSMA. PSMA-based PET imaging. In patients with advanced prostate cancer, it may also be used to enhance therapeutic effects of PSMA directed theranostics.[10,11]

In vitro data have demonstrated that PSMA expression is androgen receptor (AR)-dependent.[12] Upregulation of PSMA expression has been reported following androgen deprivation using abiraterone, enzalutamide, and ARN-509 (apalutamide).[13–15] A first case report has recently shown a sevenfold increase in uptake of ^{68}Ga-PSMA-11 detected by PET imaging 4 weeks after initiation of bicalutamide treatment in a man with castration sensitive metastatic prostate cancer.[15] On the other hand, downregulation of PSMA expression has been reported using dihydrotestosterone and vitamin D3.[16] These initial results underline the promising concept of pharmacologically induced PSMA overexpression for improved prostate cancer imaging and therapy.

We hypothesized that a significant alteration of PSMA expression is also possible using other approved compounds. Therefore, five commonly used pharmacological compounds (dutasteride, rapamycin, metformin, lovastatin, acetylsalicylic acid [ASA]) with proven antitumor effect in vitro via AR down-regulation or alternative pathways were tested for their influence on the PSMA expression and compared to the effect induced by controls (enzalutamide). Androgen receptor expressing LNCaP cells, a well established PSMA expressing in vitro model for prostate cancer, and epithelial PNT1A cells, representing benign prostate tissue, were tested.[13]

2 | MATERIALS AND METHODS

2.1 | Cell culture and expansion

LNCaP (ATCC, CRL-1740) cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). LNCaP cells were selected as well established and known PSMA expressing in vitro model. PNT1A epithelial cells were a generous gift from Pirkko Härkönen (Institute of Biomedicine, University of Turku, Finnland).[17] PNT1A cells were used as in vitro model for benign prostate tissue with unknown PSMA status. Both cell lines were cultured in RPMI medium with phenol red (Life Technologies, ThermoFisher SCIENTIFIC, Waltham, MA) supplemented with 10% FBS and 1% penicillin/streptomycin (P/S) and incubated at 37°C with 5% CO_2. Cells were used not longer than 10 passages. Medium was changed twice a week.

2.2 | Pharmacological compounds

Cells were treated up to 7 days with different concentrations of enzalutamide, dutasteride, or rapamycin (all purchased from Selleckchem, Luzern, Switzerland), metformin hydrochloride, lovastatin or acetylsalicylic acid (ASA) (all obtained from Sigma-Aldrich, Buchs, Switzerland), according to the experimental conditions. The vehicle used was 0.1% dimethyl sulfoxide (DMSO). Normal medium (RPMI with phenol red) containing 10% FBS and 1% P/S was used for all experiments. Medium containing compounds was generally changed on day 3 after initial treatment. All experiments were performed up to 7 days and performed in triplicate.

2.3 | Cell growth assay

Cells were plated at a density of 5×10^3 cells per well in a 96-well plate (Costar, Corning, NY) and cultured overnight. The next day cells were treated with different drug concentrations in 100 µL media per well according to the study protocol. Cell viability was measured by WST-1 cell proliferation assay on day 1, 3, and 7 after initial drug treatment. The WST-1 reagent (Roche Applied Science, Indianapolis, IN) was used according to the manufacturer's protocol. WST-1 reagent (100 µL/mL) was added to the culture medium and then incubated with the reagent for 3 h at 37°C, with 5% CO_2. Afterward, 100 µL of developed media/reagent from each well was transferred to a new 96-well plate and absorbance was measured at 450 nm on a microplate reader AD340 (Beckman Coulter Inc., Brea, CA).

2.4 | Flow cytometry (PSMA surface staining)

Cells were incubated overnight in a 10 cm-tissue culture dish (TPP, Trasadingen, Switzerland) at a density of 5×10^3 cells/cm^2. Medium was exchanged the next day and after 4 days with or without treatment. Prior to analysis cells were washed with PBS, detached, and pelleted by centrifugation at 1400 rpm for 5 min. For PSMA surface staining cells were directly immunolabeled with human anti-PSMA/FOLH1 antibody (clone REA408) and human Isotype REA Control APC (S) (#130-104-614) both purchased from Myltenyi Biotec, Bergisch Gladbach, Germany, according to the manufacturer's protocol. After the cells were washed twice, all cells were re-suspended in PBS and kept on ice until the measurements. Cell fluorescence was measured immediately after staining with a Becton Dickinson FACS Canto Flow Cytometer and the data were analyzed with FlowJo software v. 7.5 (Tree Star Inc., Ashland, Oregon). All data were expressed as the percentage of positive cells compared to untreated control as determined by flow cytometry.

2.5 | Immunocytochemistry

Cells were seeded on chamber slides (LabTek, ThermoFisher SCIENTIFIC, Switzerland) in growth medium for 1 day. Next day cells were treated as mentioned above for 7 days. The indirect immunostainings for cells were performed at 37°C with 4 h incubation

using the primary antibodies Anti-PSMA/FOLH1 (clone 460420, 1:100, R&D Systems, Zug, Switzerland) and AR (clone D6F11, 1:500, CellSignaling, Leiden, the Netherlands). The slides were incubated with secondary antibodies: goat anti-rabbit FITC (1:500, Vector Laboratories) or Cy3-conjugated goat anti-mouse antibody (Sigma, 1:1000) at room temperature for 1 h. After counter-staining with DAPI (4′,6-diamidino-2-phenylindole, Sigma, 1:200) the slides were analyzed by confocal laser-scanning microscopy (Leica SP8 inverse microscope, Mannheim, Germany).

2.6 | Protein simple WES immunoblotting

After 7 days of culturing, cells were washed with cold PBS supplemented with a protease inhibitor cocktail (Sigma-Aldrich) and lysed with modified lysis buffer. Total protein was measured with the BCA Protein Assay Kit (ThermoFisher SCIENTIFIC, Lausanne, Switzerland). Protein at a concentration of 1 mg/ml was used for the WES sample preparation using the 12-230 kDa cartridge kit. Proteins were separated in WES with a capillary cartridge according to the manufacturer's protocols (Protein Simple WES, Germany). Primary antibodies were mouse anti-PSMA/FOLH1 (R&D Systems, 4:100) and rabbit anti-AR (CellSignaling, 1:100). Mouse anti-GAPDH (Novus Biologicals Europe, 1:100) served as internal control.

2.7 | Uptake and internalization assay

^{177}Lu-PSMA-617 was prepared as previously reported.[18] PSMA-617 (ABX GmbH, Radeberg, Germany) was labeled with ^{177}Lu (Isotope Technologies Garching [ITG GmbH], Germany) at a specific activity of 5 MBq/nmol by incubation of the reaction mixture (pH 4.5) at 95°C for 10 min. Quality control of ^{177}Lu-PSMA-617 was performed by high-performance liquid chromatography (HPLC) using a C-18 reversed-phase column. The radiochemically pure product (>98%) was diluted in saline and subsequently used for the internalization experiment with LNCaP cells. Cells were cultured under standard conditions (37°C and 5% CO_2) in T-175 cell culture flasks and treated with 1 μM enzalutamide, 5 μM dutasteride, or 0.1 μM rapamycin, respectively. The supplemented cell culture medium was changed at day 3 after splitting. At day 7, the cells were seeded in poly-lysine coated 12-well plates at a cell concentration of 1×10^6 and 2×10^6 cells per well, respectively, and allowed to adhere over night at 37°C and 5% CO_2. After washing cells once with PBS, 975 μL RPMI medium (without supplements) and 25 μL ^{177}Lu-PSMA-617 (37.5 kBq, 7.5 pmol) were added to each well. Cells were then incubated for 4 h (37°C and 5% CO_2). To determine total uptake, cells were washed three times with ice-cold PBS. The internalized fraction was determined after washing additionally with stripping buffer (0.05 M glycine stripping buffer in 100 mM NaCl, pH 2.8) to remove surface-exposed PSMA-bound radioligand.[18] All cell samples were lysed by addition of NaOH (1 M, 1 mL) to each well and measured in a γ-counter (Perkin Elmer, Wallac Wizard 1480). The protein concentration was determined for each sample using a Micro BCA Protein Assay kit (Thermo Fisher Scientific, Switzerland) in order to standardize the measured radioactivity to the protein concentration. The data obtained with both cell concentrations were combined and the relative uptake of radioactivity, as well as the internalized fraction, was expressed as percentage of the uptake determined in samples incubated with vehicle only (100%).

2.8 | Statistical analysis

Data analysis was performed using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, version 7). A one-way ANOVA with Bonferroni's multiple comparison post test was performed to determine statistical significance. *P*-values < 0.05 were considered significant. All data presented are expressed as means with corresponding standard error of the mean (±SEM).

3 | RESULTS

3.1 | Effect of different pharmacological compounds on the viability of LNCaP and PNT1A cells

To test the effect of different treatment times and concentrations of enzalutamide, dutasteride, rapamycin, metformin, lovastatin, and ASA on cell growth, initial dose finding assays were performed with LNCaP and PNT1A cells on days 1, 3, and 7 (Figure 1). In general, LNCaP cells showed increased proliferation in a time-dependent manner detected by WST-1 assay. Compared to day 1 proliferation was significantly higher at day 7 in all experimental conditions except for rapamycin and ASA treatments. In contrast, PNT1A showed no differences between the treatment groups on days 1 and 3 and proliferation was observed only on day 7.

Based on the effective concentration and time, further analysis was performed on day 7, which showed greater differences between the treatments groups. Treatment with vehicle only (0.1% dimethyl sulfoxide [DMSO]) did not alter cell growth of LNCaP and PNT1A cells (mean optical density [OD] ± SEM; 1.6 ± 0.13 and 3.0 ± 0.09) compared to corresponding cell controls (1.3 ± 0.33, 2.6 ± 0.15). All tested concentrations of enzalutamide (1, 5, 10 μM) led to a significantly reduced cell growth of LNCaP cells (0.4 ± 0.03, 0.4 ± 0.03, 0.3 ± 0.02, all $P < 0.05$). In contrast, cell growth of PNT1A cells was not influenced by enzalutamide (2.8 ± 0.14, 2.5 ± 0.04, 2.3 ± 0.21). A similar pattern of results was observed for different concentrations of dutasteride (1, 2, 5 μM), leading to a significantly reduced cell growth in of LNCaP (0.5 ± 0.09, 0.7 ± 0.06 and 0.4, all $P < 0.05$), but not of PNT1A cells. Rapamycin (0.1, 1 μM) significantly inhibited cell growth of both cell lines (LNCaP: 0.2 ± 0.001, 0.2 ± 0.004, PNT1A: 1.2 ± 0.04, 1.2 ± 0.04, all $P < 0.001$). Treatment with metformin (10, 25, 50 μM) did not significantly alter cell growth of neither cell line. Lovastatin significantly inhibited cell growth only at a concentration of 10 μM (0.7 ± 0.01, $P < 0.05$) in LNCaP cells, but at all concentrations in PNT1A cells (1 μM: 0.9 ± 0.03, 2 μM: 0.8 ± 0.01, 10 μM: 0.3 ± 0.005, all $P < 0.001$). ASA significantly inhibited cell growth at 1, 2, and 5 mM in both cell lines (LNCaP: 0.4 ± 0.04, 0.2 ± 0.003, 0.1 ± 0.001, PNT1A: 1.7 ± 0.04, 0.4 ± 0.01, 0.1 ± 0.001, all $P < 0.001$).

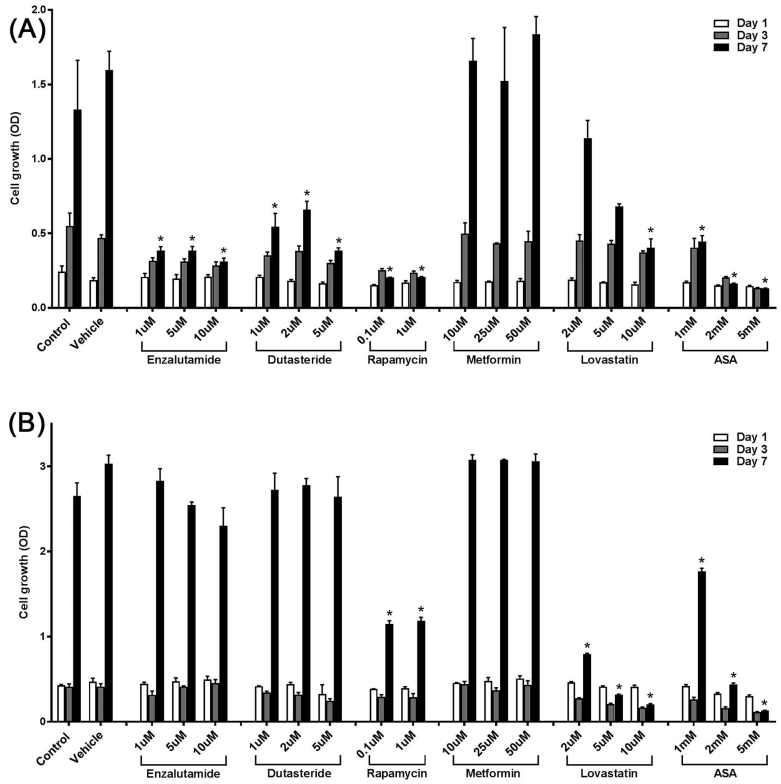

FIGURE 1 Cell viability assay: Cell growth assay measured by WST-1 assay. Cell growth was analyzed in (A) LNCaP and (B) PNT1A cells treated for 1, 3, and 7 days with different pharmacological compounds in different concentrations. Data are shown as mean with standard error of the mean (± SEM) of three to six experiments. All the treatments groups were compared to untreated control at Day 7. *Asterisks indicate statistically significant differences ($P < 0.05$)

For lovastatin and ASA the least toxic compound concentration detected by viability assay was selected for further testing. No significant different effects on cell viability were observed in the concentrations used of enzalutamide, dutasteride, rapamycin, and metformin. The lowest compound concentration was also selected for further experiments using enzalutamide and rapamycin, since these compounds show relevant adverse effects in humans. Dutasteride and metformin can be used clinically in wider dose concentrations. Thus, only for dutasteride and metformin the highest tested compound concentration was further investigated. Following tests on PSMA expression were performed with effective concentrations of 1 μM enzalutamide, 5 μM dutasteride, 0.1 μM rapamycin, 50 μM metformin, 2 μM lovastatin, and 1 mM ASA.

3.2 | Upregulation of PSMA surface levels by different pharmacological compounds in vitro

We then investigated whether different approved compounds can induce PSMA surface levels compared to the untreated cells. Therefore, PSMA surface localization was measured by flow cytometry on LNCaP and PNT1A cells treated with the previously selected concentrations of all compounds for 7 days (Figure 2). Low-dose treatment (1 μM) of enzalutamide in LNCaP cells significantly upregulated PSMA surface levels compared to baseline PSMA expression of the untreated control (Figure 2A). In contrast, PNT1A cells showed no baseline PSMA expression and no induction by low-dose treatment with enzalutamide (Figure 2B) or any other tested compound (data not shown). Therefore, further experiments to investigate PSMA expression were conducted only with LNCaP cells.

Pharmacological stimulation of PSMA expression in LNCaP cells led to a significant upregulation of PSMA surface levels after treatment with enzalutamide (206 ± 37.6%, $P < 0.05$) and dutasteride (243 ± 55.8%, $P < 0.05$). Treatment with rapamycin showed a non-significant trend toward upregulation of PSMA surface levels (183 ± 4.3%), whereas no significant changes were observed after treatment with vehicle control (0.1% DMSO), metformin, lovastatin, or ASA (84 ± 3.4%, 91 ± 4.8%, 101 ± 12.3%, and 80 ± 8.4%), respectively (Figure 2C). Further studies were conducted using enzalutamide (1 μM), dutasteride (5 μM), and rapamycin (0.1 μM).

3.3 | PSMA and AR immunocytochemistry

Consistent with the FACS results, immunostaining confirmed PSMA upregulation following treatment with enzalutamide, dutasteride and rapamycin in LNCaP cells. AR staining revealed a cytoplasmic redistribution of the AR in all compound treated cells. Treatment with vehicle control (0.1% DMSO) did not change baseline PSMA and AR expression. Staining of PNT1A cells showed no PSMA induction after treatment with enzalutamide (Figure 3A).

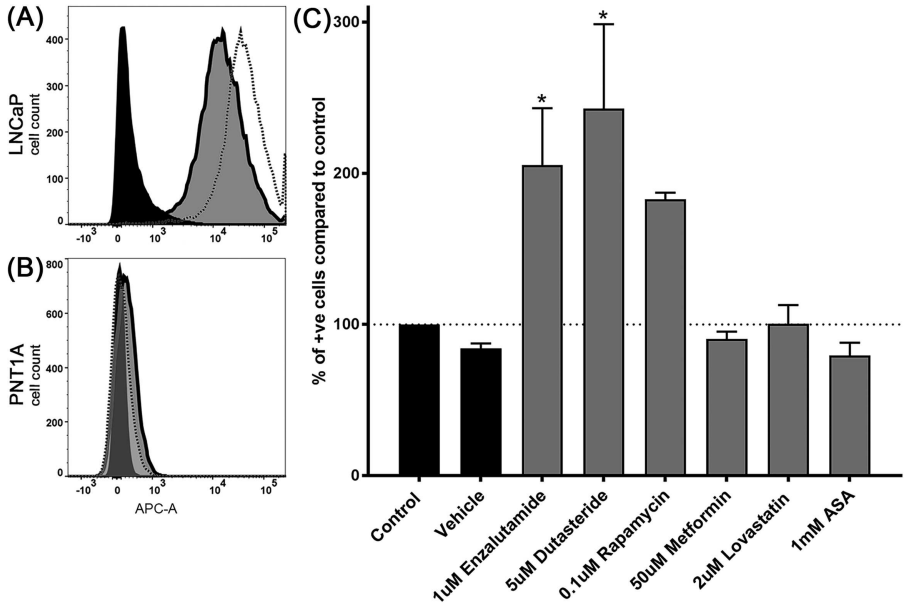

FIGURE 2 PSMA surface localization: PSMA surface localization detected by flow cytometry. Expression was measured on cells treated with different pharmacological compounds for 7 days. (A) LNCaP and (B) PNT1A cells were treated with 1 μM enzalutamide (dotted line). Filled black histograms represent background staining with isotype-control, filled gray histograms show untreated control. (C) PSMA surface levels in LNCaP cells presented as percentage of positive cells compared to untreated control. Data are shown as mean with standard error of the mean (± SEM) of four to six experiments. *Asterisks indicate statistically significant differences ($P < 0.05$)

3.4 | Total PSMA and AR protein expression

To further analyze the link between induction of PSMA expression and the AR, total PSMA and AR protein expression was measured by Western blot (WES) and normalized to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Figure 3B). Treatment with enzalutamide and rapamycin significantly increased the total PSMA expression (222 ± 15.1% and 270 ± 58.5%, both $P < 0.05$), whereas dutasteride led to a non-significant increase, of total PSMA expression (208 ± 32.4%, $P = 0.09$) compared to untreated control. Dutasteride (81 ± 33.9%), rapamycin (83 ± 10.2%), and enzalutamide (127 ± 27.1%) had no effect on AR expression (Figure 3C).

3.5 | Uptake and internalization assay

Cell uptake and internalization of ^{177}Lu-PSMA-617 were investigated in LNCaP cells treated with enzalutamide, dutasteride, rapamycin, and vehicle control. Relative to the vehicle control, cells exposed to either enzalutamide, dutasteride, or rapamycin showed a significant increase in total uptake of the radioligand after 4 h incubation time (168 ± 9.2%, 145 ± 5.4%, and 184 ± 6.4%, respectively, all $P < 0.05$). The mean internalization rate was significantly increased for cells treated with enzalutamide and rapamycin (83 ± 5.9% and 92 ± 5.3%, both $P < 0.05$), while a non-significant trend toward increased internalization was detected for the treatment with dutasteride (80 ± 6.3%, $P = 0.06$) compared to vehicle control (60 ± 4.7%). Consistent with the internalization, the calculated surface binding rate was significantly increased for cells treated with enzalutamide and rapamycin (102 ± 14.1% and 92 ± 8%, both $P < 0.05$), but not for cells treated with dutasteride (66 ± 8.8%) compared to vehicle treatment (40 ± 2.9%) (Figure 4).

4 | DISCUSSION

The present study describes the induction of PSMA expression by different commonly prescribed Food and Drug Administration (FDA) approved pharmacological compounds. Here, we demonstrate that different compounds can increase the expression of PSMA in LNCaP prostate cancer cells. Additionally, we show for the first time that dutasteride and rapamycin upregulate PSMA surface levels and ^{177}Lu-PSMA-617 uptake in vitro. These results encourage to further analyze dutasteride and rapamycin as potential inducers of PSMA surface levels prior to imaging or as chemosensitizer for therapeutic approaches.

Upregulation of total PSMA expression has been described for the first time in 1996 after culturing LNCaP cells in androgen-depleted (charcoal-stripped) media.[19] Later, Evans et al confirmed that PSMA expression is AR dependent and can be assessed quantitatively by PET imaging in vitro and in a xenograft model.[12] Treatment of VCaP cells with abiraterone, a second generation inhibitor of steroidogenesis, increased both PSMA expression and uptake of PSMA-binding tracers.[14] Murga et al later showed similar results for enzalutamide, a potent C-terminal binding AR inhibitor, in LNCaP cells. In addition, the group demonstrated that PSMA surface levels returned to baseline after withdrawal of enzalutamide, highlighting the temporal relationship of PSMA expression and AR inhibition.[13] Our results confirm upregulated PSMA surface levels and total protein expression in LNCaP cells, an established PSMA expressing in vitro model, 7 days after treatment with low-doses of enzalutamide. Moreover, we demonstrate an increased uptake and internalization of ^{177}Lu-PSMA-617 induced by enzalutamide. PNT1A epithelial prostate

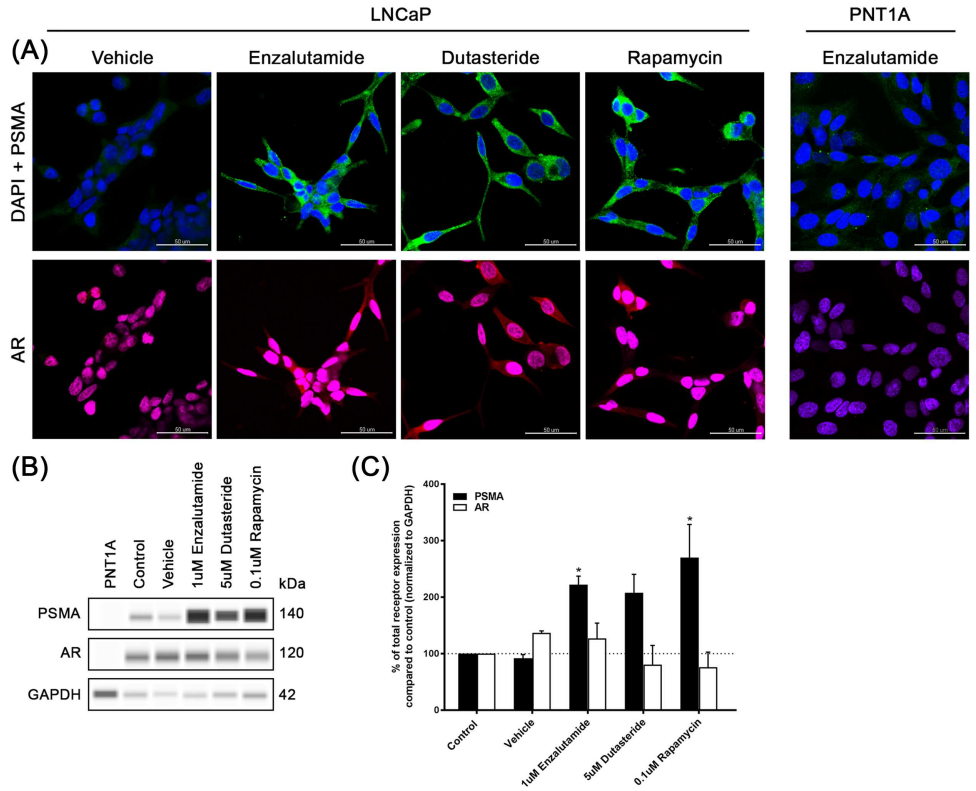

FIGURE 3 Immunocytochemistry and total protein expression. Visualization of PSMA expression using immunocytochemistry. (A) Confocal images of PSMA surface staining. LNCaP cells were cultured on chamber slides and incubated for 7 days with vehicle (0.1% DMSO), 1 μM enzalutamide, 5 μM dutasteride, or 0.1 μM rapamycin. PNT1A control was treated with 1 μM enzalutamide. Samples were stained with primary anti-PSMA antibody and detected using FITC (green) conjugated secondary antibody and DAPI (blue, 4′,6-diamidino-2-Phenylindole) In addition, staining with primary anti-AR antibody and detection using Cy3 (red) conjugated secondary antibody was performed. Scale bars indicate 50 μM. (B) Total PSMA and AR protein expression in LNCaP cells incubated for 7 days with vehicle (0.1% DMSO), 1 μM enzalutamide, 5 μM dutasteride, or 0.1 μM rapamycin compared to untreated LNCaP (control) and untreated PNT1A. Data from a representative single experiment. (C) LNCaP cells incubated as explained in (B). Percentage of total PSMA (black) and AR (white) expression in LNCaP cells treated with the above compounds (as mentioned in B) compared to control and normalized to GAPDH. Data are shown as mean with standard error of the mean (± SEM) of four to six experiments. *Asterisks indicate statistically significant differences ($P < 0.05$)

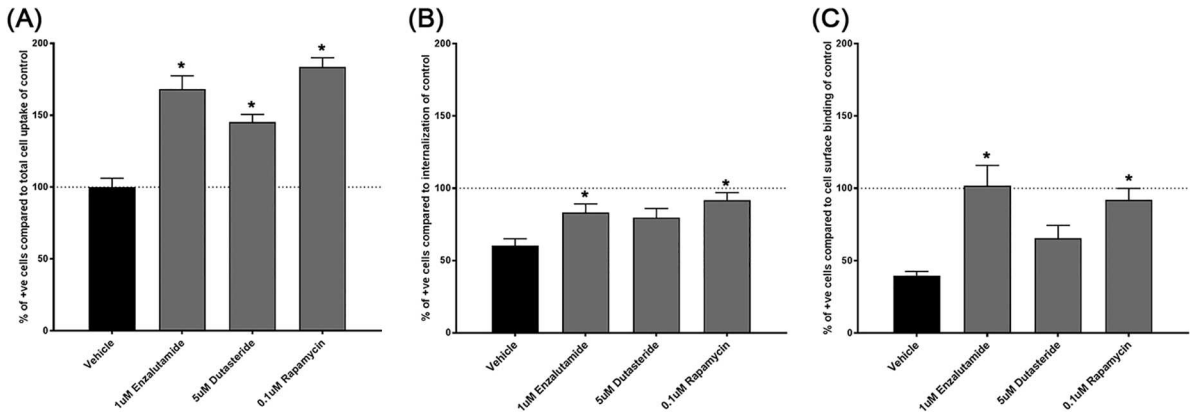

FIGURE 4 Uptake and internalization of ^{177}Lu-PSMA-617. (A) Total cell uptake, (B) internalized fraction, and (C) surface-bound fraction of ^{177}Lu-PSMA-617 in LNCaP cells after incubation for 7 days with vehicle (0.1% DMSO), 1 μM enzalutamide, 5 μM dutasteride, or 0.1 μM rapamycin. Data are shown as mean with standard error of the mean (± SEM) of four experiments performed in triplicates. *Asterisks indicate statistically significant differences ($P < 0.05$)

cells were detected to be PSMA negative, without the possibility to stimulate PSMA expression.

Dutasteride is a 5α-reductase inhibitor, blocking the conversion of testosterone to the more potent dihydrotestosterone (DHT). Dutasteride is commonly used for the conservative treatment of benign prostatic hyperplasia.[20] Lazier et al revealed that high concentrations of dutasteride promote loss of AR protein and increase cell death in LNCaP cells.[21] Chhipa et al showed that the inhibitory effect of dutasteride on AR signaling is cell line specific. Furthermore, they could demonstrate that mutations in the ligand binding domain of the AR do not significantly influence the inhibitory effect of dutasteride on the AR.[22] Additional investigations revealed a cell line specific stimulation of apoptosis by high concentrations of dutasteride in AR expressing cells.[23] Gene expression analysis showed that dutasteride not only disrupts the AR pathway but also impacts genes involved in metabolic, cell cycle and apoptotic responses in LNCaP cells.[24] Furthermore, in vitro data indicated that gene regulation by dutasteride requires the expression of the AR. Therefore, in the AR negative cell lines PC-3 and DU-145 no regulation of genes could be observed in dutasteride treated cells.[25,26] We detected a strong inhibitory effect of dutasteride on LNCaP cell growth, independent of different concentrations (1-5 μM). No inhibitory effect was seen in epithelial prostate cells (PNT1A). Furthermore, we describe, for the first time, increased PSMA surface levels, an increased total protein expression as well as uptake of ^{177}Lu-PSMA-617 induced by dutasteride treatment in LNCaP cells.

Further drugs tested in our study include rapamycin, a well described mammalian target of rapamycin (mTOR) inhibitor generally used for immunosuppression after solid organ transplantation. In vitro data revealed a post-transcriptional regulation of the AR through mTOR.[27] Wang et al reported a stimulation of the AR transcriptional activity and an impaired induction of apoptosis by rapamycin single treatment.[28] Murga et al described upregulated PSMA surface levels after treatment with rapamycin in C4-2, but not in LNCaP cells. AR expression was not significantly altered by rapamycin in their study.[13] Our findings confirm a non-significantly changed total AR protein expression in LNCaP cells after 7 days of treatment with rapamycin. Furthermore, we detected a significant upregulation of PSMA surface levels and total protein expression underlined by an increased uptake and internalization of ^{177}Lu-PSMA-617.

In addition to enzalutamide, dutasteride and rapamycin, we also tested metformin, lovastatin and ASA for their capability to induce PSMA expression. In the literature, down-regulation of full-length AR protein expression was shown after treatment of LNCaP cells with metformin and lovastatin.[29,30] Metformin also down-regulated the AR splice variant AR-V7 in CWR22Rv1 cells.[29] A direct effect of salicylate or ASA (pro-drug) on the AR expression has not been reported. However, O'Brien et al revealed that salicylate activates the activated protein kinase (AMPK) and that the inhibitory effect of salicylate on prostate cancer cells is largely based on de novo lipogenesis.[31] Interestingly, our investigations could not reveal any upregulation of PSMA surface expression after treatment of LNCaP with metformin, lovastatin, or ASA. Metformin neither reduced cell growth in LNCaP nor in PNT1A cells, whereas lovastatin and ASA showed strong inhibition of cell growth by any of the tested concentrations.

Further studies are warranted to identify and better understand factors and pathways influencing the PSMA regulation in prostate cancer. The short-term use of low-dose androgen deprivation therapy (ADT) or the use of high-dose non-ADT compounds in order to upregulate PSMA expression has several clinical implications for imaging and therapy of prostate cancer. Especially patients with low PSA values (<0.5 ng/mL) at biochemical recurrence might profit from increased tumor detection rates. Recent data demonstrated improved outcome when salvage radiotherapy (sRT) to the pelvis was performed at PSA values <0.2 ng/mL.[32–34] However, in up to 30% of all treated patients, sRT does not affect PSA levels, most likely due to non-visible extrapelvic localization of the recurrence.[35] Improved tumor detection rates might lead to clinically relevant changes in patient management. In addition, therapeutic effects of PSMA targeted theranostics might increase in patients with advanced prostate cancer after PSMA upregulation.[10,36]

5 | CONCLUSION

In conclusion, our data provide new insights into the effect of different approved pharmacological compounds that can markedly upregulate PSMA expression in vitro. Enzalutamide, dutasteride, and rapamycin significantly increase the uptake of ^{177}Lu-PSMA-617 in LNCaP cells. These findings encourage to further study these compounds. Pharmacological upregulation of PSMA expression may prove useful to improve prostate cancer detection and to enhance anticancer effects in PSMA-based radioligand therapy.

CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

AUTHORS' CONTRIBUTIONS

BK, SS participated in the design of the study, performed the experiments, analyzed the results, and drafted the manuscript. CAU, CM performed and analyzed the experiments using ^{177}Lu-PSMA-617 and critically revised the manuscript. TS, IAB critically revised the manuscript. DE participated in the design of the study, analyzed the results, and critically revised the manuscript.

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