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ORIGINAL ARTICLE

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Concentration-dependent effects of dutasteride on prostate-specific membrane antigen (PSMA) expression and uptake of ^{177}Lu-PSMA-617 in LNCaP cells

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Abstract

Background: Prostate-specific membrane antigen (PSMA)-based imaging and therapy are increasingly used in the management of prostate cancer. However, low PSMA surface expression in certain patients is a limitation for PSMA-based technologies. We have previously shown that high doses of dutasteride, a 5α-reductase inhibitor generally used for the treatment of benign prostatic enlargement, increase the PSMA expression in vitro. We now further analyzed the concentration- and time-dependent effects of dutasteride in LNCaP cells.

Methods: Androgen receptor (AR) expressing prostate cancer cells (LNCaP) were treated for 7 to 14 days with vehicle control (0.1% dimethyl sulfoxide) or different concentrations of dutasteride (0.25 , 0.5 , 1 , and 5 μM). In addition to cell proliferation, PSMA surface expression was assessed using flow cytometry (FACS) and immunocytochemistry. Total PSMA and AR expression was analyzed by capillary western immunoassay (WES). In addition, tumor cell uptake and internalization assays of ^{177}Lu-PSMA-617 were performed.

Results: Dutasteride treatment resulted in a significant upregulation of PSMA surface expression compared to vehicle control after 7 days in all tested concentrations. After 14 days a further, concentration-dependent increase of PSMA surface expression was detectable. Total PSMA protein expression significantly increased after treatment of cells with high concentrations of dutasteride using 5 μM for 7 or 14 days. However, when lower concentrations were used total PSMA expression was not significantly altered compared to vehicle control. Further testing revealed a dose-dependent increase in uptake and internalization of ^{177Lu}-PSMA-617 after 7 and 14 days. Though, a significantly increased uptake was only observed using a 5 μM dutasteride concentration for 7 days as well as 1 and 5 μM for 14 days.

Conclusion: Our investigations revealed a concentration- and time-dependent effect of dutasteride on PSMA expression and uptake of ^{177Lu}-PSMA-617 in LNCaP cells. A short-term treatment of patients with high doses of dutasteride might increase the detection rate of PSMA-based imaging and increase the effect of ^{177Lu}-PSMA-617 therapy via upregulation of PSMA expression.

KEYWORDS
^{177Lu}-PSMA-617, dutasteride, prostate cancer, prostate-specific membrane antigen

1 | INTRODUCTION

Prostate-specific membrane antigen (PSMA)-based diagnostics using ^{68}Ga-PSMA-11 as a radioligand for positron emission tomography (PET) imaging improved the detection of primary and recurrent prostate cancer.[1] Since ^{68}Ga-PSMA-11 PET imaging showed a significantly improved detection rate of local recurrence in particular patients with biochemical recurrence after primary curative treatment seem to benefit from this novel imaging modality.[2] In addition, PSMA-based therapy has been investigated as a treatment option for patients with advanced castration-resistant prostate cancer (CRPC). Several pilot studies using ^{177}Lu-PSMA-617 radioligand therapy showed a relevant antitumor activity and promising response rates in patients with CRPC.[3,4] Prospective randomized clinical trials with long-term follow-up are awaited to confirm the clinical utility of the novel treatment option.

However, these new diagnostic and therapeutic modalities are based on an elevated intratumoral PSMA expression, and despite these improvements, the detection rate of ^{68}Ga-PSMA-11 PET imaging in patients with low volume metastatic disease is still limited.[5,6] Pharmacologically inducing PSMA overexpression might be able to improve the detection rate of ^{68}Ga-PSMA-11 PET imaging and to increase the antitumor activity of ^{177}Lu-PSMA-617 radioligand therapy.

Recent studies demonstrated an increased PSMA expression following treatment with bicalutamide, abiraterone, and enzalutamide in vitro and in vivo.[7-9] In addition, results of a first-in-human application have shown a seven-fold increase in PSMA radioligand uptake following treatment with bicalutamide and a single injection of leuprolide acetate.[10] Furthermore, studies suggested a time-dependent effect of androgen receptor (AR) inhibition using enzalutamide on PSMA expression.[7] Our group has previously demonstrated that PSMA expression can also be upregulated using high concentrations of dutasteride in vitro.[11] Dutasteride is a 5α-reductase inhibitor with a well tolerable risk profile, widely used for the treatment of benign prostatic enlargement.[12] Dutasteride inhibits 5α-reductase-isoenzymes type 1 and 2 regulating the synthesis of dihydrotestosterone from testosterone. Dual inhibition of 5α-reductase leads to almost complete suppression of serum dihydrotestosterone. So far, dutasteride is the only commonly prescribed compound with a low toxicity profile that has shown to influence PSMA expression in vitro. Given that dutasteride is a potential candidate to pharmacologically induce PSMA overexpression before PSMA-based diagnostics or therapy, we aimed to analyze dose- and time-dependent effects of dutasteride on PSMA expression in LNCaP cells.

2 | MATERIALS AND METHODS

2.1 | Cell culture

LNCaP (CRL-1740; American Type Culture Collection [ATCC]) cells obtained from (ATCC, Manassas) were cultured in Roswell Park Memorial Institute (RPMI) with phenol red (Life Technologies, Thermo Fisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (P/S). Cells were incubated at 37°C with 5% CO_2 and treated for 7 to 14 days with vehicle 0.1% dimethyl sulfoxide (DMSO) or different concentrations of dutasteride (0.25, 0.5, 1, 5 μM) a 5α-reductase inhibitor (Selleckchem, Luzern, Switzerland). Cell culture medium containing compounds was generally changed twice a week. All experiments were performed in triplicate.

2.2 | WST-1 cell proliferation 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, 7, and 14 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 hours 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 Colter Inc, Brea, CA).

2.3 | Fluorescence-assisted cell sorting

Cells were cultured overnight in dishes (TPP, Trasadingen, Switzerland) at a density of 5×10^3 cells/cm^2. The medium was exchanged the next day and vehicle or dutasteride was added. Before analysis cells were washed with phosphate-buffered saline (PBS), detached and pelleted by centrifugation at 1400 rpm for 5 minutes. 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 Miltenyi Biotec (Bergisch Gladbach, Germany) according to the manufacturer's protocol. After the cells were washed twice, all cells were resuspended 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.

2.4 | Immunocytochemistry

Cells were seeded on chamber slides (LabTek, Thermo Fisher Scientific, Switzerland) in growth medium for 1 day. Next day cells were treated as mentioned above for 7 or 14 days. The indirect immunostainings for cells were performed at 37°C with

4 hours incubation using the primary antibodies Anti- PSMA/FOLH1 (clone 460420; 1:100; R&D Systems, Zug, Switzerland). The slides were incubated with secondary antibody: goat antirabbit fluorescein isothiocyanate (1:500, Vector Laboratories) at room temperature for 1 hour. After counter-staining with 4′,6-diamidino-2-phenylindole (DAPI; 1:200; Sigma) the slides were analyzed by confocal laser-scanning microscopy (Leica SP8 inverse microscope, Mannheim, Germany).

2.5 | Protein simple immunoblotting

Cells were washed with cold PBS and lysed with modified lysis buffer supplemented with a protease inhibitor cocktail (Sigma-Aldrich, Switzerland). Total protein was measured with the BCA Protein Assay Kit (Thermo Fisher Scientific, Lausanne, Switzerland). Protein at a concentration of 1 mg/mL was used for the capillary western immunoassay (WES) sample preparation using the 12 to 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 (4:100; R&D Systems) and rabbit anti-AR (1:100; CellSignaling). Mouse anti-GAPDH (1:100; Novus Biologicals Europe) served as an internal control.

2.6 | Uptake and internalization of ^{177}Lu-PSMA-617

^{177}Lu-PSMA-617 was prepared as previously reported.[13] PSMA-617 (ABX GmbH, Radeberg, Germany) was labeled with ^{177}Lu (n.c.a. ^{177}Lu, ITG GmbH, Germany) at a specific activity of 5 MBq/nmol at pH 4.5. Quality control of ^{177}Lu-PSMA-617 was performed by high-performance liquid chromatography 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. At day 7, the cells were seeded in polylysine coated 12-well plates and allowed to adhere overnight. After washing cells once with PBS, ^{177}Lu-PSMA-617 (37.5 kBq, 7.5 pmol) in RPMI medium was added to each well. Cells were then incubated for 4 hours (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 the cells with glycine buffer (pH 2.8) to remove surface-exposed PSMA-bound radioligand.[13] All cell samples were lysed (NaOH1 M, 1 mL) and measured in a γ-counter (Perkin Elmer, Wallac Wizard 1480). The protein concentration was determined using a micro BCA Protein Assay kit (Pierce, Thermo Fisher Scientific) to standardize the measured radioactivity to the protein concentration. The data of three experiments were combined and the relative uptake and internalized fraction, respectively, were expressed as a percentage of the uptake determined in samples incubated with vehicle only (100%).

2.7 | Statistical analysis

Data analysis was performed using GraphPad Prism (GraphPad Software, Inc, La Jolla, CA, version 7). A one-way analysis of variance with Bonferroni's multiple comparison post-test was performed to determine statistical significance. $P < .05$ were considered significant. All data presented are expressed as means with the corresponding standard error of the mean (± SEM).

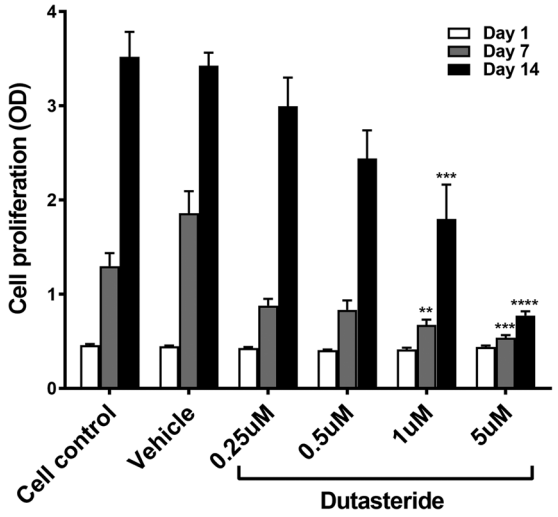

FIGURE 1 Cell proliferation was assessed by WST-1. LNCaP cells were treated for 1, 7, or 14 days with vehicle control (0.1% DMSO) or different concentrations of dutasteride (0.25, 0.5, 1, and 5 μM). Data is shown as mean with standard error of the mean (± SEM) of six independent experiments. DMSO, dimethyl sulfoxide. All treatment groups were compared to untreated control: *$P < .05$, **$P < .01$, ***$P < .001$, ****$P < .0001$

3 | RESULTS

3.1 | Cell proliferation

Initial experiments were performed to analyze the concentration-dependent effects of dutasteride on cell proliferation after 7 and 14 days using WST-1 assays (Figure 1). No relevant effect on cell proliferation was observed on day 1. Cell control (OD ± SEM; day 7: 1.3 ± 0.1, day 14: 3.5 ± 0.3) and vehicle control (day 7: 1.9 ± 0.2, day 14: 3.4 ± 0.1) demonstrated a time-dependent cell proliferation. On day 7 dutasteride treated samples showed a reduced cell proliferation (0.25 μM: 0.9 ± 0.1, 0.5 μM: 0.8 ± 0.1, 1 μM: 0.7 ± 0.1, and 5 μM: 0.5 ± 0.02) compared to vehicle control. A significant reduction of cell proliferation was only observed for higher concentrations of dutasteride using 1 μM ($P < .01$) or 5 μM ($P < .001$), respectively. On day 14 after treatment dutasteride led to reduced cell proliferation in a dose-dependent manner compared to vehicle control (0.25 μM: 3.0 ± 0.3, 0.5 μM: 2.4 ± 0.3, 1 μM: 1.8 ± 0.4, and 5 μM: 0.8 ± 0.04). Similar to the result on day 7, a significant reduction of cell proliferation was only noticed for a dutasteride concentration of 1 μM ($P < .001$) or 5 μM ($P < .0001$) on day 14.

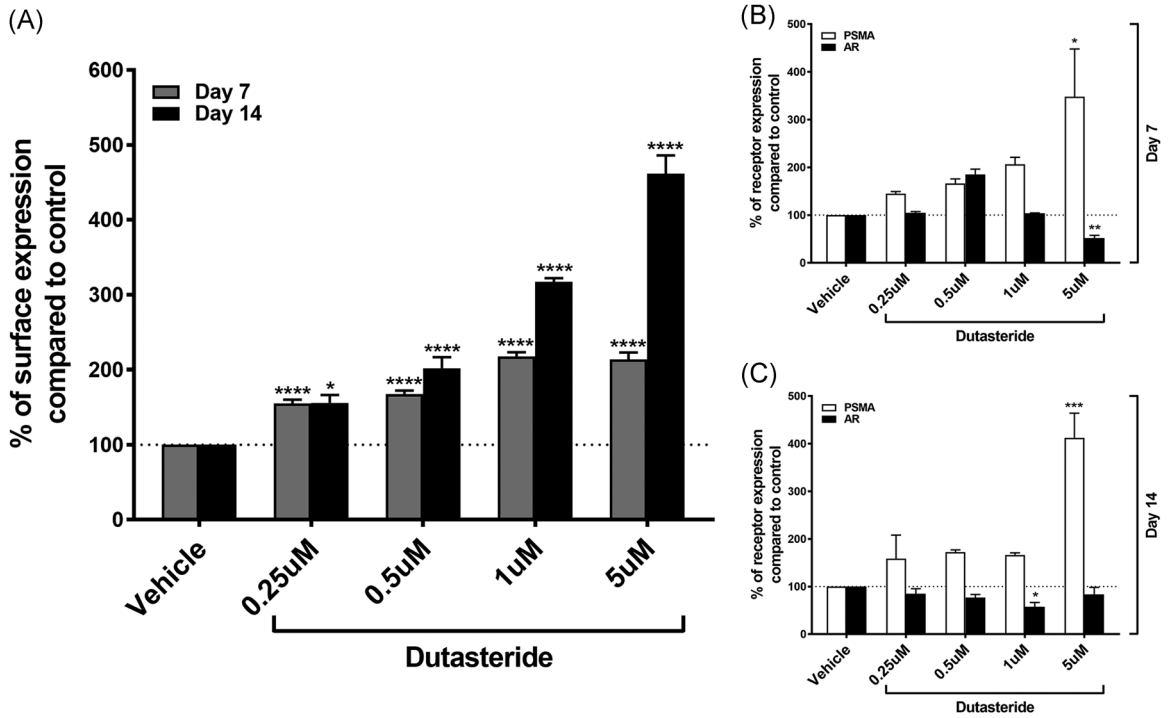

FIGURE 2 PSMA and AR expression, PSMA expression analyzed by fluorescence assisted cell sorting and protein simple immunoblotting. PSMA surface expression (A) was measured on cells treated for 7 or 14 days with vehicle control (0.1% DMSO) or different concentrations of dutasteride (0.25, 0.5, 1, and 5 μM). Total PSMA and AR protein expression was measured on cells treated for 7 (B) or 14 days (C) using the above-mentioned conditions. PSMA and AR expression is presented as the percentage of positive cells compared to vehicle control. Data is shown as mean with standard error of the mean (± SEM) of three to six independent experiments. AR, androgen receptor; PSMA, prostate-specific membrane antigen. *$P < .05$, **$P < .01$, ***$P < .001$, ****$P < .0001$

3.2 | Dutasteride induced PSMA surface expression

PSMA surface expression was measured using fluorescence-assisted cell sorting analysis. According to our initial experiments, PSMA surface expression was analyzed on day 7 and 14 after treatment with four different concentrations of dutasteride (0.25, 0.5, 1, 5 μM), and compared to treatment with vehicle control (Figure 2A). Dutasteride significantly upregulated PSMA surface expression in all tested concentrations (0.25 μM: 155% ± 5%, 0.5 μM: 168% ± 5%, 1 μM: 218% ± 5%, and 5 μM: 213% ± 9%; all $P < .0001$) on day 7. An even higher PSMA surface expression was observed after stimulation of cells with dutasteride for 14 days (0.25 μM: 156% ± 10%; $P < .05$, 0.5 μM: 202% ± 15%, 1 μM: 317% ± 5%, and 5 μM: 461% ± 24%; for 0.5-5 μM; all $P < .0001$).

3.3 | Effect of dutasteride on total PSMA and AR protein expression

In addition to PSMA surface expression, also total PSMA and AR expression using protein simple immunoblotting was assessed. Treatment of cells with 5 μM dutasteride (348% ± 100%) led to a significant ($P < .05$) increase in total PSMA expression compared to vehicle control, whereas lower concentrations (0.25 μM: 145% ± 5%, 0.5 μM: 166% ± 10%, and 1 μM: 206% ± 15%) led only to a slight, insignificant increase on day 7 (Figure 2B).

Similar results were observed after 14 days of treatment: 0.25 μM: 159% ± 49%, 0.5 μM: 172% ± 5%, 1 μM: 166% ± 5%, and 5 μM: 412% ± 52%; $P < .001$; Figure 2C).

In contrary to PSMA, total AR expression showed no clear tendency after 7 days of treatment (0.25 μM: 105% ± 3%, 0.5 μM: 185% ± 11%, 1 μM: 104% ± 1%, and 5 μM: 52% ± 6%). A significant AR downregulation was only observed after treatment with 5 μM dutasteride ($P < .01$). After 14 days, there seemed to be a slight tendency towards an AR downregulation (0.25 μM: 85% ± 11%, 0.5 μM: 77% ± 7%, 1 μM: 58% ± 9%, and 5 μM: 84% ± 15%), which was only significant for a treatment with 1 μM dutasteride ($P < .05$).

3.4 | Dutasteride treated cells exhibit an increased PSMA expression in a dose-dependent manner detected by immunocytochemistry

Visualization of PSMA expression in LNCaP cells was performed by immunocytochemistry (Figure 3). Our results confirmed a dose-dependent upregulation of PSMA expression upon dutasteride treatment. A strong PSMA expression was observed after treatment of cells for 14 days with 1 or 5 μM dutasteride compared to vehicle control.

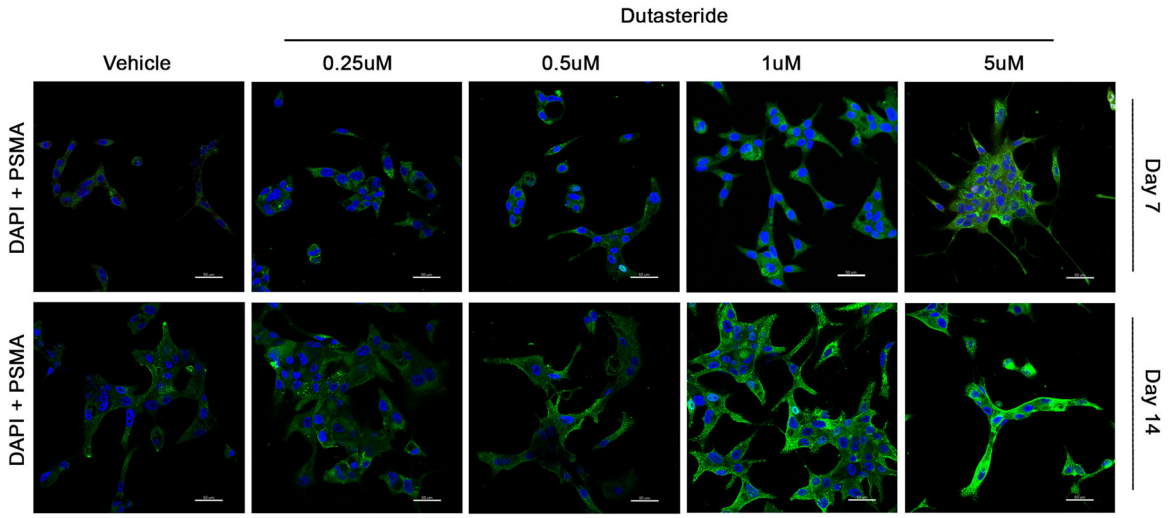

FIGURE 3 PSMA immunocytochemistry, Visualization of PSMA expression using immunocytochemistry. Confocal images of PSMA surface staining. LNCaP cells were cultured on chamber slides and incubated for 7 or 14 days with vehicle control (0.1% DMSO) or different concentrations of dutasteride (0.25, 0.5, 1, and 5 μM). Samples were stained with primary anti-PSMA antibody and detected using FITC (green) conjugated secondary antibody and DAPI (blue, 4′,6-diamidino-2-Phenylindole). Scale bars indicate 50 μM. Data from a representative single experiment. DMSO, dimethyl sulfoxide; FITC, fluorescein isothiocyanate; PSMA, prostate-specific membrane antigen [Color figure can be viewed at wileyonlinelibrary.com]

3.5 | Uptake and internalization of ^{177}Lu-PSMA-617

To further assess whether the observed PSMA upregulation leads also to an increased uptake of ^{177}Lu-PSMA-617, we performed tumor cell uptake and internalization assays (Figure 4). Indeed, the uptake of ^{177}Lu-PSMA-617 in LNCaP cells was increased in a dose-dependent manner on day 7 (0.25 μM: 111% ± 5%, 0.5 μM: 128% ± 9%, 1 μM: 138% ± 13%, and 5 μM: 161% ± 19%) and day 14 (0.25 μM: 115% ± 10%, 0.5 μM: 119% ± 2%, 1 μM: 146% ± 13%, and 5 μM: 152% ± 3%; Figure 4A). However, a significantly increased uptake was only observed using a concentration of 5 μM for 7 days ($P < .05$) as well as a concentration of 1 or 5 μM for 14 days (both $P < .05$). Internalization of ^{177}Lu-PSMA-617 increased in parallel to the uptake of ^{177}Lu-PSMA-617 (Figure 4B). In contrast to our uptake measurements, internalization was not significantly increased after treatment of cells with 5 μM for 14 days.

4 | DISCUSSION

Our investigations revealed concentration- and time-dependent effects of dutasteride on PSMA expression and uptake of ^{177}Lu-PSMA-617 in LNCaP cells. Dutasteride treatment with all tested concentrations led to a significant upregulation of PSMA surface expression after 7 and 14 days. A 14 days treatment with 1 or 5 μM dutasteride resulted in a remarkable 3- to 4.6-fold higher PSMA surface expression compared to vehicle control, and a significantly increased uptake of ^{177}Lu-PSMA-617.

PSMA is a type II membrane glycoprotein. Its DNA has been sequenced by the laboratory of Israeli et al[14] after the initial development of the prostate cancer cell line LNCaP by Horoszevicz et al.[15] PSMA is a cell surface protein with a large extracellular domain. The exact function and regulatory mechanisms of PSMA are yet to be fully elucidated.[16] Recent work suggested that the enzymatic function of PSMA is cleaving glutamate, thereby activating the glutamate driven phosphoinositide 3-kinase and subsequently the mammalian target of rapamycin (mTOR) pathway.[17] PSMA expression is known to be upregulated up to a thousand-fold higher in prostate cancer compared to normal prostate tissue. Thus, PSMA represents a potential target for imaging and therapy of prostate cancer.[18]

However, due to low volume disease, the detection rate of PSMA-based imaging is still limited in certain patients.[6] In addition, the antitumor activity of ^{177}Lu-PSMA-617 radioligand therapy might be improved after inducing PSMA expression. Recent data suggests that androgen deprivation therapy increases PSMA expression under certain circumstances.[19] Thus, upregulation of PSMA expression following androgen withdrawal has already been described in 1996 by Wright et al.[20] In addition, Meller et al[9] revealed an increased PSMA expression following short-term treatment of prostate cancer cells with abiraterone. Further investigations suggested a time-dependent effect of AR inhibition using enzalutamide on PSMA expression in vitro.[7] Later, Evans et al[21] showed that enzalutamide increases PSMA expression also in vivo and that these changes can be quantitatively measured by using PET imaging. These results led to a first promising patient report of PSMA upregulation detected by ^{68}Ga-PSMA-11 PET/MRI following 4 weeks of treatment with bicalutamide and a single injection of leuprolide acetate.[10] On the other hand, recently published in vivo data did not show a synergistic treatment effect by using enzalutamide as pretreatment of PSMA-directed radioligand therapy.[8] Therefore, further research is required to better understand whether short-term exposure to androgen deprivation therapy might be useful to enhance imaging quality or therapy effects in prostate cancer. However, first (bicalutamide) or second (abiraterone, enzalutamide) generation androgen deprivation therapy is associated with relevant side effects in patients.

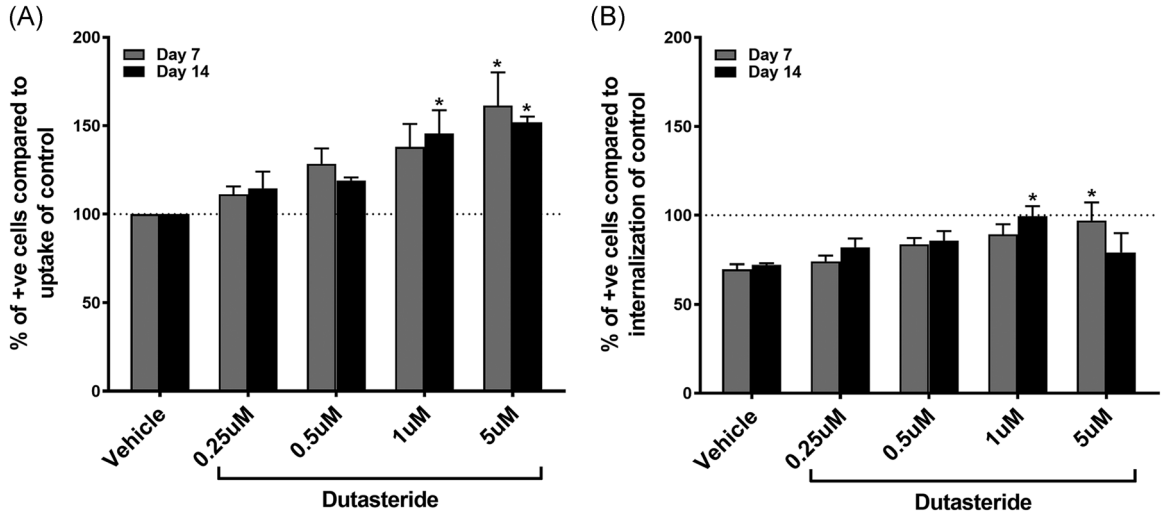

FIGURE 4 Uptake and internalization of ^{177}Lu-PSMA-617, Total cell uptake (A) and internalization (B) of ^{177}Lu-PSMA-617 in LNCaP cells treated for 7 or 14 days with vehicle control (0.1% DMSO) or different concentrations of dutasteride (0.25, 0.5, 1, 5 μM). Data is shown as mean with standard error of the mean (± SEM) of four experiments performed in triplicates. DMSO, dimethyl sulfoxide; PSMA, prostate-specific membrane antigen. *P < .05, **P < .01, ***P < .001, ****P < .0001

Dutasteride is the first alternative compound with a low toxicity profile that has shown to influence PSMA expression in vitro.[11] Since dutasteride is widely used among the male population for the treatment benign prostatic enlargement, it could easily be implemented into clinics as a possible PSMA expression enhancer before PSMA-based imaging or as synergistic PSMA expression enhancer before ^{177}Lu-PSMA-617 radioligand therapy.[12] All dutasteride concentrations tested in our in vitro studies have previously been used by others in different settings.[22-25] However, the highest serum drug concentrations observed in humans reached above 1 μM in men given an oral dose of 5 mg dutasteride daily.[26] Therefore, most concentrations of dutasteride used for in vitro and in vivo experiments are significantly higher than serum levels of dutasteride observed in clinical trials.[23] Though, the clinical safety of using oral doses up to 10 mg dutasteride daily has been demonstrated by different prospective trials.[27,28] In the current study, we included four different concentrations of dutasteride (0.25, 0.5, 1, and 5 μM) well knowing that 5 μM exceeds physiologically achievable serum concentrations and that in vitro results cannot be directly applied in clinics. Thus, using 1 μM dutasteride led to a promising upregulation of PSMA surface expression as well as significantly increased uptake of ^{177}Lu-PSMA-617 after 14 days in LNCaP cells.

Additional in vitro an in vivo studies are now required to confirm our promising results and to test dutasteride in different settings. Future investigations might focus on intracellular functions of PSMA and its exact regulatory mechanisms.

5 | CONCLUSION

Our results show a concentration- and time-dependent effect of dutasteride on PSMA expression and uptake of ^{177}Lu-PSMA-617 in LNCaP cells. A short-term treatment of patients with high doses of dutasteride might increase the detection rate of PSMA-based imaging and increase the effect of ^{177}Lu-PSMA-617 therapy via upregulation of PSMA expression.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests.

AUTHOR CONTRIBUTIONS

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