Darolutamide Potentiates the Antitumor Efficacy of a PSMA-targeted Thorium-227 Conjugate by a Dual Mode of Action in Prostate Cancer Models

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ABSTRACT

Purpose: Androgen receptor (AR) inhibitors are well established in the treatment of castration-resistant prostate cancer and have recently shown efficacy also in castration-sensitive prostate cancer. Although most patients respond well to initial therapy, resistance eventually develops, and thus, more effective therapeutic approaches are needed. Prostate-specific membrane antigen (PSMA) is highly expressed in prostate cancer and presents an attractive target for radionuclide therapy. Here, we evaluated the efficacy and explored the mode of action of the PSMA-targeted thorium-227 conjugate (PSMA-TTC) BAY 2315497, an antibody-based targeted alpha-therapy, in combination with the AR inhibitor darolutamide.

Experimental Design: The *in vitro* and *in vivo* antitumor efficacy and mode of action of the combination treatment were investigated in preclinical cell line–derived and patient-derived prostate cancer xenograft models with different levels of PSMA expression.

Results: Darolutamide induced the expression of PSMA in androgen-sensitive VCaP and LNCaP cells *in vitro*, and the efficacy of darolutamide in combination with PSMA-TTC was synergistic in these cells. *In vivo*, the combination treatment showed synergistic antitumor efficacy in the low PSMA-expressing VCaP and in the high PSMA-expressing ST1273 prostate cancer models, and enhanced efficacy in the enzalutamide-resistant KUCaP-1 model. The treatments were well tolerated. Mode-of-action studies revealed that darolutamide induced PSMA expression, resulting in higher tumor uptake of PSMA-TTC, and consequently, higher antitumor efficacy, and impaired PSMA-TTC–mediated induction of DNA damage repair genes, potentially contributing to increased DNA damage.

Conclusions: These results provide a strong rationale to investigate PSMA-TTC in combination with AR inhibitors in patients with prostate cancer.

Introduction

Prostate cancer is the second most common newly diagnosed malignancy in men worldwide (1) and the second leading cause of cancer-related deaths (2). Treatment options for prostate cancer include surgery, external beam radiotherapy, androgen deprivation therapy (ADT), androgen receptor (AR) inhibition, chemotherapy, and radium-223 treatment (3–6). Although most patients initially respond well to ADT, eventually the disease becomes resistant to therapy and progresses into castration-resistant prostate cancer (CRPC; refs. 3–5). CRPC is treated with AR inhibitors, such as competitive AR antagonists or androgen biosynthesis inhibitors, but unfortunately, most patients develop resistance after 18 to 24 months. Despite tremendous progress in the treatment of advanced prostate cancer in the past decade, metastatic CRPC (mCRPC) remains largely incurable, and novel therapeutic options, including combination treatments, are needed.

Enzalutamide and apalutamide are AR inhibitors that have demonstrated efficacy in pivotal phase III clinical trials in patients with different stages of prostate cancer, including CRPC (3, 5). The novel AR inhibitor darolutamide (7) has recently been approved by the FDA for the treatment of nonmetastatic CRPC, and it is currently undergoing clinical investigations in patients in other clinical settings, such as metastatic castration-sensitive prostate cancer (3, 5, 8, 9).

Prostate-specific membrane antigen (PSMA; *FOLH1*) has been shown to be an excellent target for prostate cancer treatment because of its high expression in prostate cancer cells at all stages of the disease (10–12). New treatment options are arising from PSMA-targeted radionuclide therapy using both antibody and peptide-based approaches. A number of early clinical trials with PSMA-targeting ligands labeled with beta-particle–emitting or alpha-particle–emitting radionuclides have already shown very promising results (12).

We have previously described the development and preclinical characterization of the PSMA-targeted thorium-227 conjugate (PSMA-TTC) BAY 2315497, consisting of a human anti-PSMA antibody covalently linked to a 3,2-HOPO (hydroxypyridinone) chelator moiety radiolabeled with the alpha-particle emitter thorium-227. PSMA-TTC showed strong antitumor efficacy in preclinical prostate cancer models (13), and a phase I clinical trial in patients with mCRPC is currently ongoing (ClinicalTrials.gov ID: NCT03724747).

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Translational Relevance

Most patients with prostate cancer undergoing antiandrogen treatment will develop resistance, and thus, novel effective treatments are needed. Here, we explore the combination of the antibody-based targeted alpha-therapy, PSMA-targeted thorium-227 conjugate (PSMA-TTC), and the androgen receptor inhibitor darolutamide in preclinical prostate cancer models. This combination is well tolerated and exhibits synergistic antitumor efficacy. We demonstrate a dual mode of action for this combination treatment, where androgen inhibition (i) induces PSMA expression, resulting in increased PSMA-TTC tumor uptake and enhanced efficacy in a model with low baseline PSMA expression, and (ii) prevents the expression of DNA repair pathway genes, leading to increased DNA damage and antitumor efficacy in combination with PSMA-TTC in a model with high baseline PSMA expression. Altogether, our preclinical data suggest that PSMA-TTC and darolutamide combination therapy may be a highly effective treatment option for prostate cancer and support the clinical development of PSMA-TTC in combination with androgen receptor inhibitors, such as darolutamide.

Here, we report the preclinical characterization of the antibody-based targeted alpha-therapy (TAT) PSMA-TTC in combination with the AR inhibitor darolutamide. We assessed the antitumor efficacy and mode of action of this combination treatment in preclinical prostate cancer models with different levels of endogenous PSMA expression. Taken together, the data support the clinical development of PSMA-TTC in combination with androgen inhibitors for the treatment of prostate cancer.

Materials and Methods

Compounds

The PSMA-targeted thorium-227 conjugate PSMA-TTC (BAY 2315497) consisting of the alpha-particle emitter thorium-227 complexed to a 3,2-HOPO chelator covalently linked to a fully human PSMA-targeting antibody was synthesized at Bayer AG and Bayer AS as described previously (13). Darolutamide was synthesized at Orion Corporation. Enzalutamide, the murine anti-PSMA mAb J591 and the synthetic androgen agonist R1881 were synthesized at Bayer AG.

Cancer cell lines and patient-derived xenograft models

VCaP, C4-2, and 22Rv1 prostate cancer cells were obtained from ATCC, LAPC-4 prostate cancer cells from VTT (VTT Technical Research Centre of Finland Ltd., Espoo, Finland), and LNCaP, DU-145, and PC3 prostate cancer cells from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). Cell lines were regularly subjected to DNA fingerprinting at DSMZ and tested to be free from *Mycoplasma* contamination using MycoAlert (Lonza) directly before use. The cells were routinely cultivated according to the manufacturer's protocols at 37°C in a humidified atmosphere containing 5% CO_2. For the *in vitro* experiments, LNCaP and VCaP cells were supplemented with 0.1 nmol/L of the synthetic androgen agonist R1881. The patient-derived xenograft (PDX) prostate cancer model ST1273 (14) was obtained from South Texas Accelerated Research Therapeutics and KUCaP-1 (15) from O. Ogawa (University of Kyoto, Kyoto, Japan). The LuCaP 86 and LuCaP 58 PDX models were obtained from E. Corey (University of Washington, Seattle, WA).

In vitro combination assay with PSMA-TTC and darolutamide

The *in vitro* antiproliferative activity of treatment combinations was assessed by determination of combination indexes in VCaP and LNCaP human prostate cancer cells. VCaP cells were treated with fixed-ratio combinations of PSMA-TTC (0.048–50 kBq/mL) and darolutamide (36.6 nmol/L–37.5 μmol/L). LNCaP cells were treated with fixed-ratio combinations of PSMA-TTC (0.020–20 kBq/mL) and darolutamide (36.6 nmol/L–37.5 μmol/L). Cell viability was measured using the CellTiter-Glo assay (Promega) after a 4-day exposure to PSMA-TTC. Darolutamide was added 3 days before PSMA-TTC. EC_{50} values were calculated from duplicate values for each individual combination data point, and the respective isobolograms were generated. Combination indexes (CIs) were calculated according to the median-effect model of Chou-Talalay (16) with CI < 0.8 defined as synergistic effect.

RNA isolation and quantitative PCR for *PSMA* and *CDKN1A*

Forty-eight hours after treatment of VCaP and LNCaP cells with 2 μmol/L darolutamide and 5 kBq/mL PSMA-TTC as monotherapy or combination therapy, total RNA was isolated using the RNeasy Plus Mini Kit (Qiagen) protocol followed by cDNA synthesis using the SuperScript III First-Strand Synthesis SuperMix (Thermo Fisher Scientific) for qRT-PCR. Gene expression analysis was performed using the TaqMan Fast Advanced Master Mix (Applied Biosystems) and the following TaqMan probes: *PSMA* (*FOLH1*; Hs00379515_m1), *CDKN1A* (Hs00355782_m1), and *GAPDH* (Hs02758991_g1), all from Applied Biosystems. All measurements were performed in duplicate. *PSMA* and *CDKN1A* expression were determined as fold change compared with vehicle using the $2^{-\Delta\Delta C_t}$ method and *GAPDH* as the reference gene.

Cell surface PSMA expression in VCaP and LNCaP cells

VCaP and LNCaP cells were cultured in the presence of 0 to 5 μmol/L darolutamide or enzalutamide for 10 days. Cell surface PSMA expression was determined by flow cytometry analysis (BD FACSCanto II Cell Analyzer) using the murine anti-PSMA mAb J591. The number of antigens bound per cell was determined using the QIFIKIT quantitative analysis kit (Agilent Dako) and analyzed using the FlowJo_v10.6.1 software. DMSO was used as a baseline control.

In vivo studies in various CDX and PDX prostate cancer models

PSMA expression was evaluated in the ST1273, KUCaP-1, LuCaP 86, and LuCaP 58 patient-derived (PDX) and VCaP, LNCaP, DU145, PC3, LAPC-4, C4-2, and 22Rv1 cancer cell line–derived (CDX) prostate cancer xenograft models using the mouse monoclonal PSMA antibody GCP-04 (0.02 μg/mL; Novus Biologicals; see Supplementary Materials and Methods for details).

The antitumor efficacy of PSMA-TTC and darolutamide as monotherapy and in combination was evaluated in the VCaP, ST1273, and KUCaP-1 xenograft models.

PSMA-TTC was formulated in 30 mmol/L citrate supplemented with 50 mg/mL sucrose, 70 mmol/L NaCl, 0.5 mg/mL para-aminobenzoic acid, 2 mmol/L EDTA, and 0.1 mg/mL IgG2a mAb. Darolutamide was formulated in PEG400/propylene glycol/5% glucose (50:30:20) and enzalutamide in 5% benzyl benzoate/95% peanut oil. In all PSMA-TTC and darolutamide/enzalutamide combination studies, the vehicle control group was treated with a combination of the respective formulation buffers. In the KUCaP-1 efficacy study, 0.9% saline was used as vehicle for PSMA-TTC.

To circumvent unspecific uptake of the test compound by organs, all mice were predosed intravenously with 200 μg of an irrelevant mouse

antibody (IgG2a, κ isotype, murine myeloma monoclonal UPC10 antibody, Sigma-Aldrich) 16 to 24 hours prior to treatment with PSMA-TTC (17).

In the VCaP efficacy study, male CB17-Scid mice (6 weeks, 22 ± 2 g, Janvier Labs) were inoculated subcutaneously with 3×10^6 VCaP cells suspended in 100% Matrigel. To assess the antitumor efficacy of PSMA-TTC in combination with darolutamide, mice were randomized into control and treatment groups ($n = 12$) when VCaP tumors reached an average size of 180 mm^3. VCaP tumor-bearing mice were treated with vehicle, a single intravenous injection of 150 or 300 kBq/kg PSMA-TTC at a total antibody dose of 0.14 mg/kg, darolutamide (100 mg/kg, twice daily, orally) or their combinations.

In the ST1273 efficacy study, female NMRI nude mice (7 weeks, 27 ± 3 g, Janvier Labs) were implanted subcutaneously with testosterone rods (MedRod 75 μg/day, drug release duration 100 days, PreclinApps) and, 2 days later, with $5 \times 5 \times 5$ mm^3 ST1273 tumor fragments. In the first ST1273 efficacy study, the antitumor efficacy of an intermediate PSMA-TTC dose in combination with varying doses of darolutamide was evaluated. When the tumors reached an average size of 230 mm^3, the mice were randomized ($n = 10$ mice/group) and treatment with vehicle, PSMA-TTC (250 kBq/kg at a total antibody dose of 0.14 mg/kg, single dose, intravenous), darolutamide [15, 30, or 100 mg/kg, (twice daily)× 21, orally] or their combinations was started. In the second ST1273 efficacy study, the antitumor efficacy of a fixed darolutamide dose in combination with different doses of PSMA-TTC was evaluated. When the tumors reached an average size of 180 mm^3, the mice were randomized ($n = 10$ mice/group) and treated with PSMA-TTC (75, 125, or 250 kBq/kg, all at a total antibody dose of 0.14 mg/kg, single dose, intravenous), darolutamide [100 mg/kg, (twice daily) × 21, orally) or their combinations. In a third efficacy study, the antitumor efficacy of PSMA-TTC was assessed in combination with the AR inhibitor enzalutamide. When the tumors reached an average size of 280 mm^3, the mice were randomized ($n = 10$ mice/group) and treated with PSMA-TTC (250 kBq/kg, all a total antibody dose of 0.14 mg/kg, single dose, intravenous), enzalutamide [30 mg/kg, (once daily)× 28, orally) or their combinations.

In the KUCaP-1 efficacy study, male CB17-Scid mice (8 weeks, 20 g, Janvier Labs) were implanted subcutaneously with $5 \times 5 \times 5$ mm^3 KUCaP-1 tumor fragments. When the tumors reached an average size of 100 mm^3, mice were randomized ($n = 10$ mice/group) and treated with vehicle, PSMA-TTC [150 kBq/kg at a total antibody dose of 0.43 mg/kg, (every 2 weeks) × 2, intravenous), darolutamide [200 mg/kg, (once daily) × 33] or their combination.

The *in vivo* biodistribution of PSMA-TTC was evaluated in the VCaP model. Isolated VCaP tumors were analyzed for remaining thorium-227 radioactivity (= biodistribution) 66 hours post-dosing and expressed as percentage (%) of injected dose of thorium-227 per gram (% ID/g), as described previously (18). Furthermore, isolated VCaP and ST1273 tumors were analyzed for *PSMA* RNA expression and VCaP tumors for protein expression of PSMA, phosphorylated Chk2 (pChk2) and phosphorylated Chk1 (pChk1). Briefly, RNA was isolated using a QiagenTissueLyser II and the Rneasy Plus Mini Kit (Qiagen). *PSMA* RNA expression was determined as described in the *in vitro* part. Total Chk2, pChk2, pCHk1, and PSMA protein expression in VCaP tumor lysates was analyzed by Western blotting using antibodies from Cell Signaling Technology (No. 6334 for CHk2, No. 2197 for pChk2, No. 2348 for pCHk1) and OriGene (No. TA504509 for PSMA) and quantified using the Peggy Sue instrument (ProteinSimple).

For hematology, blood samples were collected from the sublingual or jugular vein one day before the dosing of the animals in the second ST1273 study, followed by sampling on day 28 and at study end (day 49). The EDTA anticoagulated samples were analyzed by flow cytometry using the ProCyte Dx Hematology Analyzer.

Animal experiments were performed under the national animal welfare laws in Germany and Denmark and approved by the local authorities. Subcutaneous tumor growth was monitored by measuring tumor volume ($0.5 \times \text{length} \times \text{width}^2$) using a caliper. Animal body weight was monitored as an indicator of treatment-related toxicity. Measurement of tumor volume and body weight was performed two to three times per week. Individual animals were sacrificed when showing >20% body weight loss or when tumors reached a maximum volume of approximately 1,000 mm^3. At study termination, the animals were sacrificed by cervical dislocation under CO_2 anesthesia. T/C (treatment/control) ratios were calculated using final mean tumor volume at study or control group termination. Treatment responses in the ST1273 prostate cancer PDX model were defined on day 100 using the RECIST (19). Progressive disease (PD) was defined as greater than 20% increase in tumor size. Partial response (PR) was defined as greater than 30% reduction in tumor size. Complete response (CR) was defined as an absence of any palpable tumor mass. No tumor growth or a slight reduction (<30%) or small increase (<20%) in tumor size was defined as stable disease (SD).

In vivo mode-of-action—RNA sequencing, gene set enrichment analysis, and γH2AX level in ST1273 tumor xenografts

In the first ST1273 study, tumors were harvested 72 hours after treatment with 250 kBq/kg PSMA-TTC alone or in combination with darolutamide (100 mg/kg, twice daily), and RNA was isolated and purified using the RNeasy-Plus Mini Kit (Qiagen). Libraries were prepared using the Illumina TruSeq Stranded mRNA Kit and sequenced on a HiSeq 2500 HTv4 instrument (single-read, dual-indexing, 50 cycles; Illumina). For data analysis, RNA sequencing (RNA-seq) reads were aligned to the human reference genome HG38 using the STAR aligner software (version 2.5.3a), and gene expression was quantified using the RSEM software (version 1.3.0; ref. 20). Differential gene expression analysis was performed using the DESeq2 package (version 1.26.0; ref. 21) for the R Project for Statistical Computing (version 3.6.3; https://www.r-project.org/; ref. 22) by comparing mRNA expression to untreated control samples for each treatment. Genes were defined as differentially expressed with absolute $\log_2$ fold changes >1 and false discovery rates (FDRs) < 0.1. Gene set enrichment analysis (GSEA) was performed using the fast gene set enrichment analysis (fgsea) package for R (version 1.10.1; ref. 23) with $\log_2$ fold changes as input and utilizing the hallmark gene sets (24) HALLMARK_DNA_REPAIR and HALLMARK_ANDROGEN_RESPONSE from the Molecular Signatures Database v7.1 (25). Multiple testing correction for GSEA was performed across all conditions using the Benjamini–Hochberg correction.

The kinetics of drug treatment effects on the level of phosphorylated histone protein H2AX (γH2AX) were evaluated in tissue sections of paraffin-embedded ST1273 tumors (from the first ST1273 study) from untreated mice ($n = 3$) or mice treated with PSMA-TTC (250 kBq/kg) alone or in combination with darolutamide (100 mg/kg, every day, orally) for 72, 168, or 336 hours. Sections were fixed at 4°C for 5 minutes, air dried, washed with ddH_2O, and incubated with Dako S2023 Peroxidase-Blocking Solution (Agilent Technologies) at room temperature for 10 minutes. Next, the sections were washed with TBS and incubated with the mouse anti-phospho-histone H2A.X (Ser139) antibody (1:500; clone JBW301, Merck Millipore) at room temperature for 60 minutes, followed by incubation with the horseradish peroxidase–labeled anti-mouse polymer (Dako). Immunoreactions

were visualized using 3,3′-diaminobenzidine as a substrate. Positive γH2AX signals were quantified using the HS Analysis Webkit tool (HS Analysis GmbH) and plotted in the GraphPad Prism software.

Statistical analyses

Statistical analysis was performed using the statistical programming language R [R version 3.6.3 (22)]. The validity of the model assumptions was checked for the fitted statistical model. All data except the assessment of γH2AX in ST1273 tumor tissue were analyzed using linear models estimated with generalized least squares with a separate variance term for each group. Pairwise comparisons were performed using the estimated linear model and corrected for familywise error rate using either Dunnett or Sidak method where appropriate. Synergy in the *in vivo* data was determined using 50,000 Monte Carlo simulations with the estimated parameters of the fitted statistical model. In each simulation, a random value was drawn for each group by using the point estimate and its SEM and SD of a normal distribution. Synergy is found when the effect for the combo group is greater than the expected additive effect. Confidence in synergy is the proportion of simulations where synergy was found. The γH2AX data were analyzed using a linear model with a single variance term for all groups and timepoints. Mean comparisons were corrected using Sidak method.

Results

Darolutamide and PSMA-TTC combination shows synergistic effects *in vitro*

First, we explored the effect of the combination of PSMA-TTC and darolutamide *in vitro* in androgen-sensitive, low PSMA-expressing VCaP and androgen-sensitive, high PSMA-expressing LNCaP prostate cancer cells. Isobolographic analysis of cell viability data indicated that the interaction of PSMA-TTC with darolutamide was strongly synergistic both in VCaP (**Fig. 1A**) and LNCaP (**Fig. 1B**) cells with combination indexes of 0.36 and 0.47, respectively. One plausible mechanism for the observed synergy could be upregulation of PSMA by AR inhibitors as described previously (26–28). The treatment of VCaP cells with darolutamide resulted in an over 10-fold elevated expression of the *PSMA* gene compared with untreated cells (**Fig. 1C**). Also, in LNCaP cells, despite very high baseline *PSMA* levels, treatment with darolutamide led to elevated *PSMA* expression (**Fig. 1D**). Correspondingly on the protein level, 2.5 μmol/L darolutamide upregulated the cell surface PSMA expression in both VCaP and LNCaP cells (**Fig. 1E** and **F**) as indicated by nine- and eightfold increases in comparison with baseline control, respectively. Similar results were obtained with 2.5 μmol/L enzalutamide with six- and sevenfold upregulation of PSMA surface expression in VCaP and LNCaP cells, respectively. Furthermore, in both cell lines, treatment with PSMA-TTC alone or in combination with darolutamide induced the expression of *CDKN1A* ($p21^{Cip1}$), a gene linking DNA damage to cell-cycle arrest, as described previously (Supplementary Fig. S1A and S1B; ref. 13). In VCaP cells, *CDKN1A* induction was slightly higher upon combination treatment than with PSMA-TTC monotherapy.

Darolutamide induces PSMA expression and shows synergistic effects with PSMA-TTC in VCaP prostate cancer xenografts

On the basis of the observed synergistic effects *in vitro*, we next explored the antitumor efficacy of PSMA-TTC in combination with darolutamide in prostate cancer xenograft models with different baseline levels of PSMA expression (Supplementary Fig. S2A). In the VCaP model with low and heterogeneous PSMA expression (Supplementary Fig. S2A and S2B), monotherapy with a single injection of 150 or 300 kBq/kg PSMA-TTC or twice-daily treatment with 100 mg/kg darolutamide showed no significant antitumor efficacy at the given doses with T/C values of 0.92, 0.77, and 0.87, respectively (**Fig. 2A** and **B**). However, combination therapy with 150 or 300 kBq/kg PSMA-TTC and darolutamide inhibited tumor growth compared with vehicle and the corresponding monotherapies with T/C values of 0.56 and 0.55, respectively. Analysis of tumor volume indicated a synergistic combination effect for 150 or 300 kBq/kg PSMA-TTC in combination with darolutamide with 99% and 90% confidence levels, respectively (**Fig. 2A**). Even stronger evidence for synergism was obtained on the basis of tumor weight data with 99% confidence levels for both combinations (**Fig. 2B**). Interestingly, when darolutamide was administered either as a single agent or in combination with PSMA-TTC, increased PSMA expression was observed both on the RNA (**Fig. 2C**) and protein (**Fig. 2D**) level. In line with this, an almost threefold increase of thorium-227 uptake was observed in VCaP tumors upon PSMA-TTC and darolutamide combination therapy compared with PSMA-TTC alone (**Fig. 2E**). Furthermore, the combination treatment increased the phosphorylation of the DNA damage response pathway molecule Chk2 (pChk2; **Fig. 2F**), but not that of Chk1 (pChk1, Supplementary Fig. S3), indicating alpha-particle–induced formation of DNA double-strand breaks (DSB) in cancer cells. No changes in the body weights were observed in VCaP tumor-bearing mice in any of the groups (data not shown).

PSMA-TTC shows synergistic antitumor efficacy in combination with darolutamide in the ST1273 PDX prostate cancer model *in vivo*

Next, we evaluated the *in vivo* efficacy of the PSMA-TTC and darolutamide combination in the high PSMA-expressing (Supplementary Fig. S2A and S2C), hormone-dependent, castration-, and enzalutamide-sensitive (14) ST1273 prostate cancer PDX model. In the first ST1273 efficacy study, the antitumor efficacy of a fixed, intermediate PSMA-TTC dose (250 kBq/kg) in combination with varying doses of darolutamide (15, 30, or 100 mg/kg, twice daily) was evaluated. Long-lasting inhibition of tumor growth was achieved with a single injection of PSMA-TTC at an intermediate dose of 250 kBq/kg (**Fig. 3A**; **Table 1**). Darolutamide monotherapy at 15, 30, or 100 mg/kg (twice daily) resulted in tumor growth control under treatment; however, tumors started to regrow after day 20, the last day of darolutamide treatment. Combination treatment with each tested darolutamide dose and PSMA-TTC resulted in almost complete tumor eradication and a synergistic effect (99.8%–99.9% confidence on day 33, **Fig. 3B**; **Table 1**). Enhanced response rates were observed in all combination groups even after 100 days, as indicated by complete responses in 7/9, 7/9, and 9/10 mice upon treatment with PSMA-TTC and 15, 30, or 100 mg/kg (twice daily) darolutamide, respectively (**Table 1**). To limit potential side effects of PSMA-TTC therapy, we explored whether the dose of PSMA-TTC in the combination treatment could be reduced and still maintain the antitumor efficacy of the combination. Therefore, in the second ST1273 study, the efficacy of a fixed darolutamide dose (100 mg/kg, twice daily) in combination with low to intermediate doses of PSMA-TTC (75, 125, or 250 kBq/kg) was tested. At all doses, PSMA-TTC in combination with 100 mg/kg (twice daily) darolutamide showed enhanced antitumor efficacy and a synergistic effect (92.3%–100% confidence on day 19; **Fig. 3C** and **D**). Furthermore, similar synergistic effects were observed when ST1273 tumors were treated with PSMA-TTC in combination with another AR inhibitor, enzalutamide (Supplementary Fig. S4). A dose-dependent and, importantly, transient reduction of the white blood cell count was observed in mice treated with PSMA-TTC

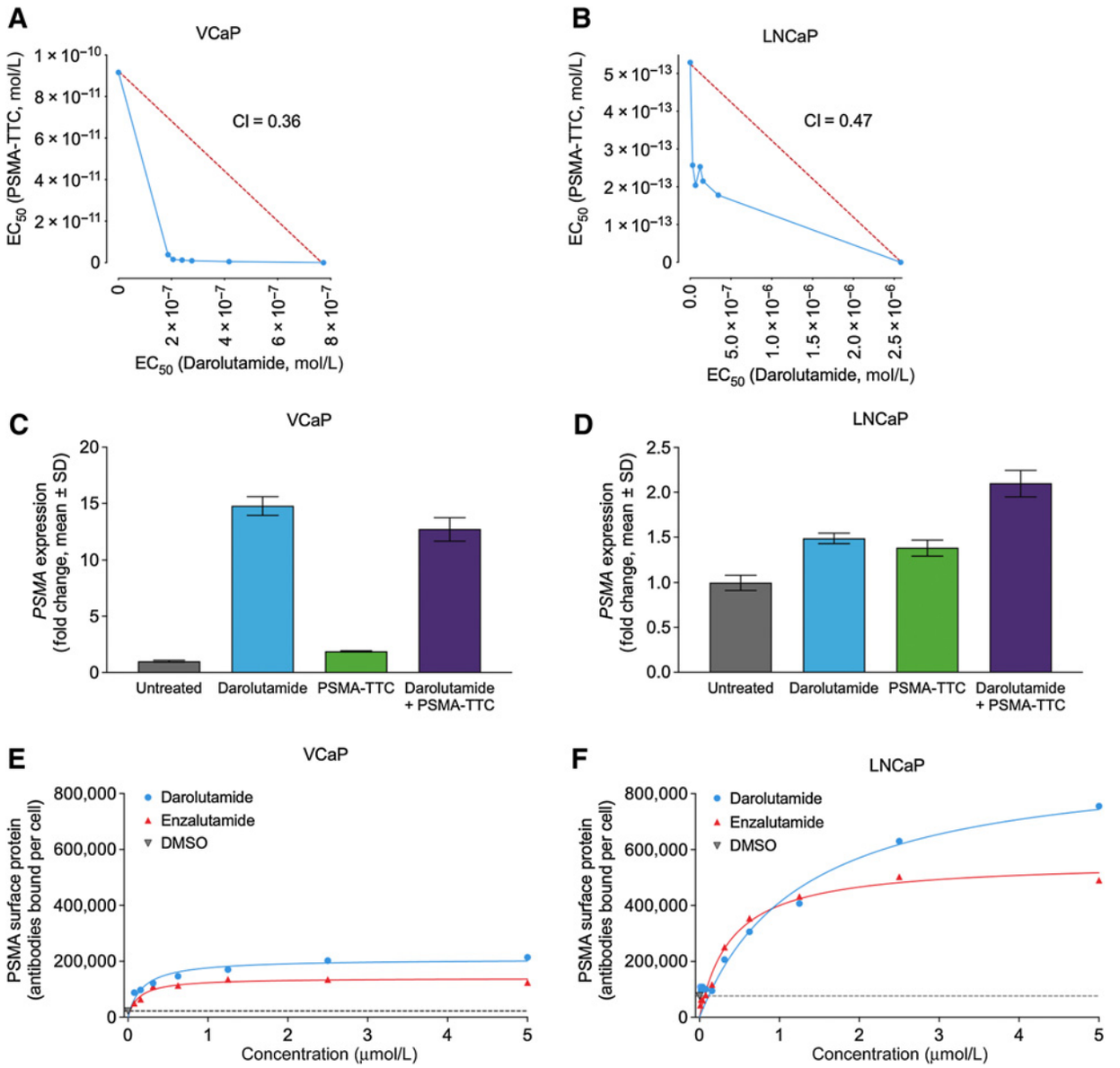

Figure 1.

Darolutamide and PSMA-TTC combination shows synergistic antitumor efficacy *in vitro*. Isobolograms for the *in vitro* combination effect of PSMA-TTC and darolutamide on the proliferation of VCaP (**A**) and LNCaP (**B**) prostate cancer cells. *PSMA* (*FOLH1*) expression in VCaP (**C**) and LNCaP (**D**) cells treated with 5 kBq/mL PSMA-TTC and/or 2 μmol/L darolutamide as determined by qRT-PCR 48 hours after treatment ($n = 2$). The expression is presented as fold change compared with untreated cells. Cell surface PSMA expression as determined by flow cytometry in darolutamide or enzalutamide-treated VCaP (**E**) and LNCaP (**F**) cells. DMSO served as a baseline control and is depicted with a dashed line. CI, combination index; DMSO, dimethyl sulfoxide.

(Supplementary Fig. S5). No body weight loss was observed in any of the treatment groups, indicating that the treatments were well tolerated (Supplementary Fig. S6A and S6B).

Darolutamide shows enhanced antitumor efficacy in combination with PSMA-TTC in the KUCaP-1 PDX prostate cancer model *in vivo*

Finally, we evaluated the *in vivo* antitumor efficacy of the PSMA-TTC and darolutamide combination treatment in the KUCaP-1 prostate cancer PDX model which is known to be resistant to the androgen inhibitor enzalutamide due to the AR mutation W742C (29). In this model, PSMA-TTC monotherapy (150 kBq/kg) showed efficacy with a T/C ratio of 0.30 and resulted in a partial response in one of 10 mice. Darolutamide monotherapy (200 mg/kg, once daily) was efficacious with a T/C ratio of 0.47 but later disease progression occurred in 10 of 10 mice (**Fig. 4A** and **B**; Supplementary Table S1). Compared with the monotherapies, the combination of PSMA-TTC with darolutamide showed enhanced antitumor efficacy with a T/C ratio of 0.10 and partial responses in four of nine mice and stable disease in one of nine mice up to 33 days after the start of treatment (**Fig. 4A** and **B**;

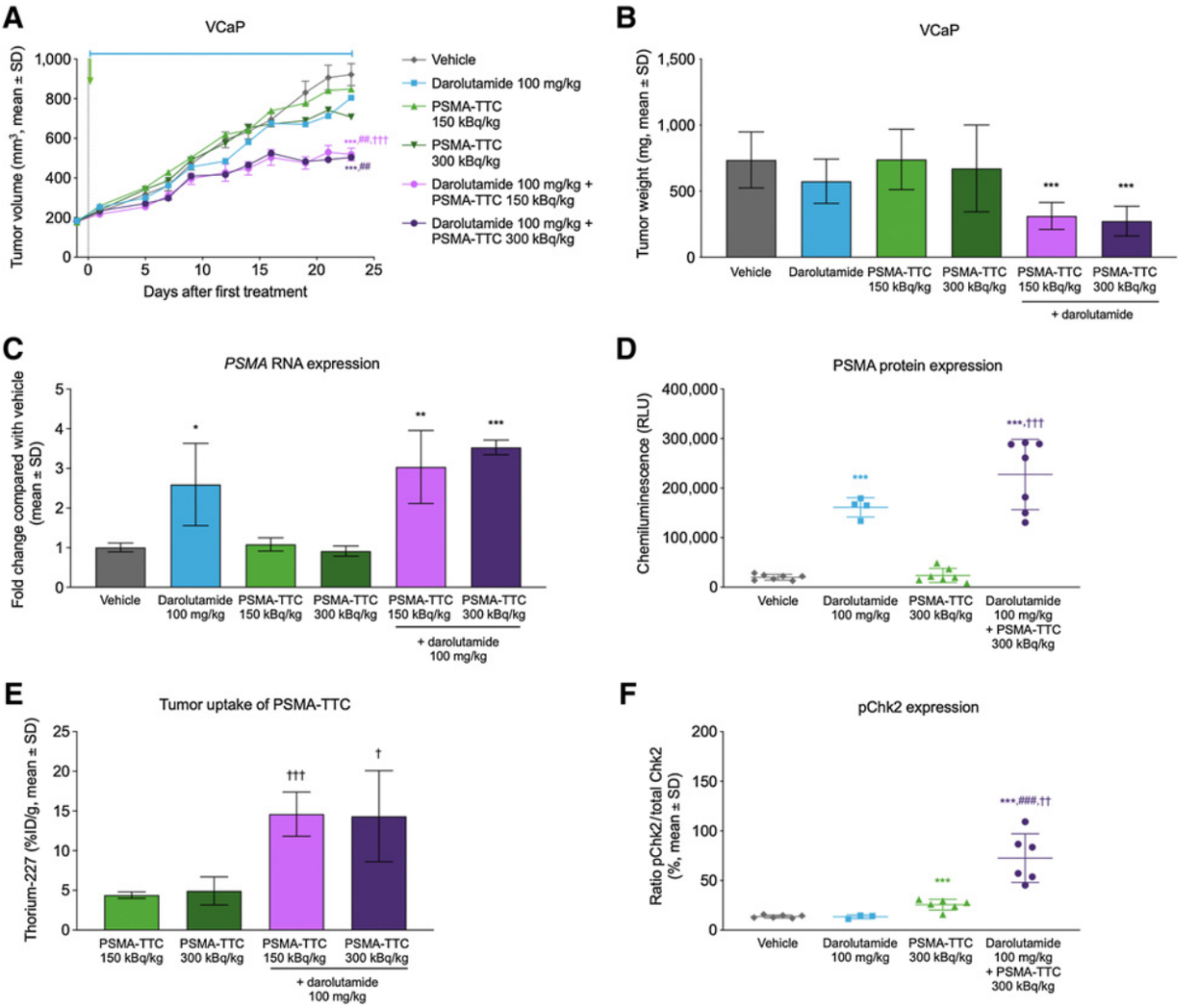

Figure 2.

Darolutamide induces PSMA expression and shows synergistic antitumor efficacy with PSMA-TTC in the VCaP prostate cancer model. Male CB17-Scid mice were treated with vehicle, a single dose of 150 or 300 kBq/kg PSMA-TTC (intravenous, total protein dose 0.14 mg/kg, treatment day indicated with a green arrow) and/or 100 mg/kg darolutamide [(twice daily) × 23, orally, treatment period indicated with a blue bar]. **A,** Growth curves of VCaP tumors ($n = 11$–12). **B,** VCaP tumor weight at the end of the study ($n = 11$–12). **C,** *PSMA* RNA expression in VCaP tumors as determined by qRT-PCR ($n = 2$–4). **D,** PSMA protein expression in VCaP tumors as determined by immunoblotting ($n = 4$–7). **E,** Thorium-227 accumulation in VCaP tumors 66 hours after dosing ($n = 4$). **F,** Expression of phosphorylated Chk2 (pChk2) in VCaP tumors as determined by immunoblotting ($n = 3$–6). Signals from pChk2 were normalized to total Chk2. Statistical analyses were performed using linear models followed by Dunnett or Sidak method. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ in comparison with vehicle. ##, $P < 0.01$; ###, $P < 0.001$ in comparison with the corresponding darolutamide monotherapy. †, $P < 0.05$; ††, $P < 0.01$; †††, $P < 0.001$ in comparison with the corresponding PSMA-TTC monotherapy.

Supplementary Table S1). No relevant decreases in body weight compared with vehicle-treated mice were observed in the KUCaP-1 model in any of the groups (data not shown).

Darolutamide impairs PSMA-TTC–mediated induction of DNA repair gene expression in the ST1273 PDX prostate cancer model

To investigate the *in vivo* mode of action of the PSMA-TTC and darolutamide combination treatment and the effects of PSMA-TTC monotherapy and combination therapy with darolutamide in a PSMA high-expressing model, gene expression signatures in ST1273 tumors were analyzed by mRNA sequencing 72 hours after treatment with 250 kBq/kg PSMA-TTC alone or in combination with 100 mg/kg (twice daily) darolutamide and compared with tumors from untreated control animals. Overall, this analysis identified 47 downregulated and 85 upregulated genes upon PSMA-TTC monotherapy, and 271 downregulated and 93 upregulated genes upon PSMA-TTC and darolutamide combination therapy. Noteworthily, the high baseline PSMA levels in the ST1273 model were not significantly altered by darolutamide treatment (Supplementary Fig. S7). Furthermore, GSEA was utilized to identify MSigDB hallmarks associated with mRNA expression changes induced by the two different treatments. PSMA-TTC treatment alone showed a low FDR of 0.004 but, as expected, did not exert a consistent effect on the expression of androgen-dependent genes (**Fig. 5A**), whereas in combination with darolutamide, strong and consistent downregulation of androgen-dependent genes was observed (FDR = 0.0014; **Fig. 5A**). Interestingly,

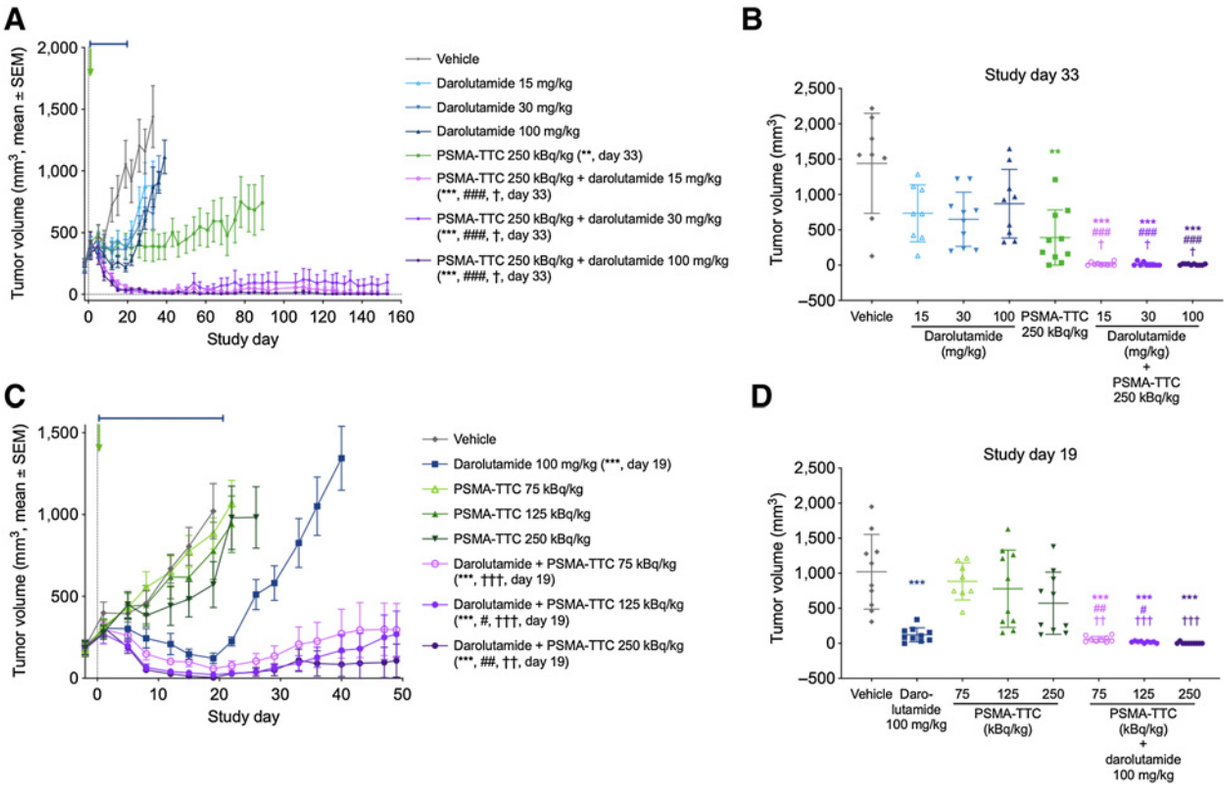

Figure 3.

PSMA-TTC and darolutamide show synergistic antitumor efficacy in the ST1273 prostate cancer PDX model. **A,** Growth curves of ST1273 tumors in female NMRI nude mice ($n = 9$–10) treated with vehicle, 250 kBq/kg PSMA-TTC (total protein dose 0.14 mg/kg, intravenous, treatment day indicated with a green arrow) and/or 15, 30, or 100 mg/kg darolutamide [(twice daily)× 21, orally; treatment period indicated with a blue bar]. **B,** Tumor volumes of individual ST1273 tumors shown in **A** on day 33. **C,** Growth curves of ST1273 tumors in female NMRI nude mice ($n = 10$) treated with vehicle, 75, 125, or 250 kBq/kg PSMA-TTC (intravenous, total protein dose 0.14 mg/kg; treatment day indicated with a green arrow) and/or 100 mg/kg darolutamide [(twice daily)× 21, orally; treatment period indicated with a blue bar]. For the generation of the mean tumor volume curves in **A** and **C**, the last measured tumor volume values of mice which were euthanized due to large tumor size were included into the calculation of the mean value in the graph until $n \geqq 8$ (last study day shown in graph). **D,** Tumor volumes of individual ST1273 tumors shown in **C** on day 19. Statistical analyses were performed on day 33 or day 19 for the studies shown in **A** and **C**, respectively, using the estimated linear model followed by Sidak method. **, $P < 0.01$; ***, $P < 0.001$ in comparison with vehicle. $^{\#}$, $P < 0.05$; $^{\#\#}$, $P < 0.01$; $^{\#\#\#}$, $P < 0.001$ in comparison with the corresponding darolutamide monotherapy. †, $P < 0.05$; ††, $P < 0.01$; †††, $P < 0.001$ in comparison with the corresponding PSMA-TTC monotherapy.

PSMA-TTC monotherapy led to marked upregulation of genes in the DNA repair hallmark gene set (FDR = 0.0026), which was not observed when PSMA-TTC was administered in combination with darolutamide (FDR = 0.29; **Fig. 5B**). A heatmap of the genes included in the DNA repair hallmark gene set demonstrated that the expression of DNA repair genes was predominantly upregulated by PSMA-TTC monotherapy (shown in red; **Fig. 5C** and more detailed in Supplementary Fig. S8). Upon combination treatment with darolutamide, downregulation of DNA repair genes was observed (shown in blue; **Fig. 5C** and more detailed in Supplementary Fig. S8). To assess the functional consequences of altered gene expression, H2AX phosphorylation (γH2AX) as a measure of DNA damage was determined in ST1273 tumors after 72, 168, or 336 hours of treatment with 250 kBq/kg PSMA-TTC alone and in combination with 100 mg/kg (twice daily) darolutamide. Highest γH2AX levels were detected in the combination group 336 hours after treatment (**Fig. 5D** and **E**). This observation may be explained by the lack of DNA repair gene expression upon combination treatment shown in **Fig. 5A**.

Discussion

Despite recent progress in the treatment of advanced prostate cancer, the disease ultimately remains incurable with poor prognosis (30). Therefore, new treatment options are highly needed. Accordingly, there has been growing interest in radionuclide therapy for CRPC with agents targeting PSMA, a transmembrane glycoprotein that is highly overexpressed on the surface of prostate cancer cells, including hormone-refractory and metastatic stages of the disease (11, 31). PSMA-targeted radiotherapy using peptide- or antibody-based approaches radiolabeled with the beta-emitter lutetium-177 (^{177}Lu) or the alpha-emitters actinium-225 (^{225}Ac) and thorium-227 (^{227}Th) are currently undergoing clinical testing (32–34). Even though preliminary efficacy and safety data on PSMA-targeted radionuclide therapy are very encouraging, the full therapeutic potential of this approach may be limited by interpatient and intrapatient heterogeneity of PSMA expression and dose-limiting side effects (35).

PSMA-directed targeted alpha-therapy with small molecule inhibitors has demonstrated clinical activity in patients refractory to

Table 1. Efficacy of PSMA-TTC, varying doses of darolutamide, and their combinations in the ST1273 prostate cancer PDX model.

Treatment	T/C ratio on day 33	Response rate on day 100[a]			
		CR	PR	SD	PD
Darolutamide 15 mg/kg[b,c]	0.60	n.a.	n.a.	n.a.	n.a.
Darolutamide 30 mg/kg[b,d]	0.45	n.a.	n.a.	n.a.	n.a.
Darolutamide 100 mg/kg[b,e]	0.60	n.a.	n.a.	n.a.	n.a.
PSMA-TTC 250 kBq/kg	0.27**	1/6	0/6	0/6	5/6
Darolutamide 15 mg/kg + PSMA-TTC 250 kBq/kg	0.01***, ###, †	7/9	1/9	1/9	0/9
Darolutamide 30 mg/kg + PSMA-TTC 250 kBq/kg	0.01***, ###, †	7/9	0/9	1/9	1/9
Darolutamide 100 mg/kg + PSMA-TTC 250 kBq/kg	0.01***; ###, †	9/10	1/10	0/10	0/10

Note: **, $P < 0.01$; ***, $P < 0.001$ in comparison with untreated control. ###, $P < 0.001$ in comparison with the corresponding darolutamide monotherapy. †, $P < 0.05$ in comparison with the corresponding PSMA-TTC monotherapy.
Abbreviation: n.a., not applicable.
[a]Responses were determined according to RECIST.
[b]Treatment period for darolutamide: study days 0–20.
[c]Last study day 33.
[d]Last study day 36.
[e]Last study day 39.

beta-radiation, highlighting the potential of alpha-particle emitters (33). However, the application of small molecule–based PSMA-targeted alpha-therapy with ^{225}Ac-PSMA-617 has been limited by severe xerostomia due to strong uptake of the PSMA-targeting ligand into the salivary glands (36). In turn, with antibody-based PSMA-targeting approaches, the observed salivary gland uptake is very low (36–38). Therefore, antibody-based targeted alpha-therapy, such as PSMA-TTC, is an attractive concept to complement the portfolio of PSMA-targeting radionuclide therapies. However, myelosuppression is a common phenomenon when using IgG-based radiotherapeutics in the clinic, and it has also been confirmed in preclinical studies with PSMA-TTC (13). Thus, combination therapy with compounds that have nonoverlapping toxicities, such as darolutamide or other AR inhibitors, is an interesting opportunity to broaden the therapeutic window of antibody-based targeted alpha-therapy.

Here, we investigated for the first time the combination of the antibody-based PSMA-TTC, which belongs to the emerging class of TATs (39), with the AR inhibitor darolutamide in preclinical prostate cancer models with different characteristics. Combination treatment with PSMA-TTC and darolutamide demonstrated synergistic efficacy in the VCaP model, characterized by low and heterogenous PSMA expression at baseline. Increased PSMA expression and synergistic cytotoxic effects on cancer cells were observed upon darolutamide treatment *in vitro*. Correspondingly, the *in vivo* combination treatment with darolutamide and PSMA-TTC resulted in increased PSMA expression and accumulation of thorium-227 in the tumor. Higher phosphorylation of Chk2, indicative of DNA damage, that is, DSBs, was observed, resulting in higher antitumor activity. Several studies have shown that androgen inhibition upregulates the expression of PSMA (26–28) and that increased tumor PSMA expression results in higher tumor uptake of PSMA-targeting drugs (40). Furthermore, increased uptake of PSMA-binding PET tracers upon androgen inhibition has been demonstrated in both preclinical prostate cancer models as well as in patients with prostate cancer (40–45). However, to our knowledge, our data demonstrates for the first time that increased PSMA expression by AR inhibition can increase the antitumor activity of a radionuclide therapy in a preclinical model of prostate cancer. The studies presented here were conducted in immunocompromised mice and additional contribution of the immune system to the antitumor efficacy remains to be elucidated.

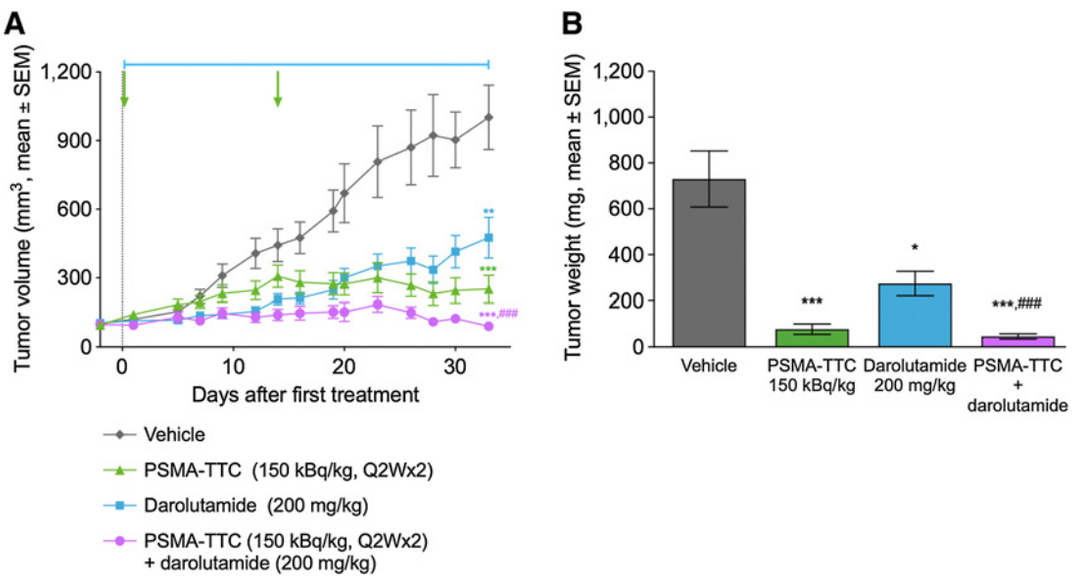

Figure 4.
Darolutamide shows enhanced antitumor efficacy in combination with PSMA-TTC in the KUCaP-1 prostate cancer PDX model. Male CB17-Scid mice ($n = 10$ mice/group) were treated with vehicle, 150 kBq/kg PSMA-TTC every 2 weeks (Q2W) × 2, intravenous, total protein dose 0.43 mg/kg, treatment days indicated with green arrows), 200 mg/kg darolutamide (once daily, orally, treatment period indicated with a blue bar) or their combination. **A,** Growth curves of KUCaP-1 PDX tumors. **B,** KUCaP-1 tumor weights at the end of the study. Statistical analyses were performed using linear models followed by Sidak method. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ in comparison with vehicle on day 33; ###, $P < 0.001$ in comparison with darolutamide monotherapy on day 33.

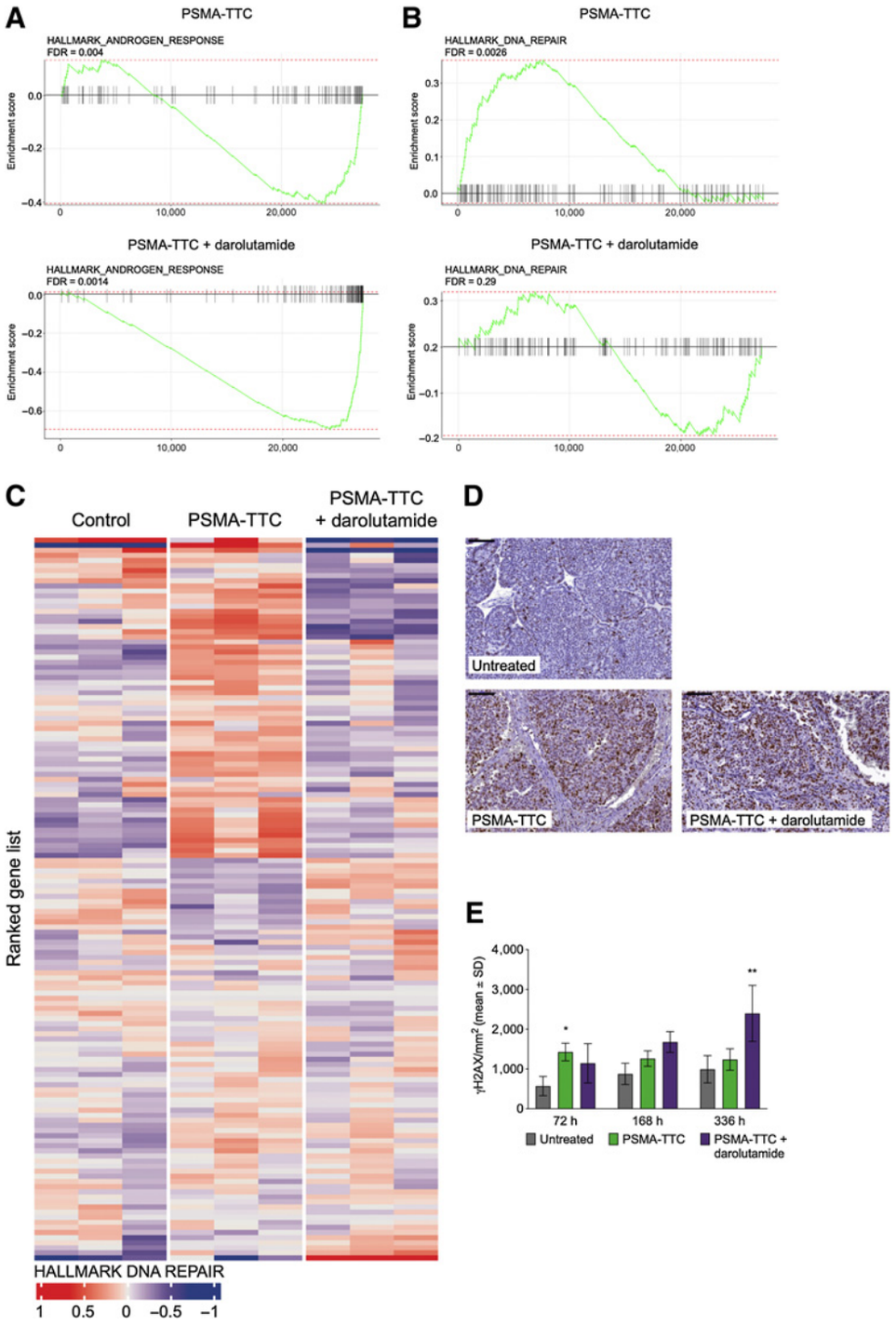

Figure 5.

Darolutamide impairs the PSMA-TTC–mediated induction of DNA repair genes in the ST1273 PDX prostate cancer model. **A,** Enrichment plot of androgen-dependent genes after treatment with 250 kBq/kg PSMA-TTC alone (FDR = 0.004) or in combination with 100 mg/kg (twice daily) darolutamide (FDR = 0.0014). **B,** Enrichment plot of DNA repair genes after treatment with 250 kBq/kg PSMA-TTC alone (FDR = 0.0026) or in combination with 100 mg/kg (twice daily) darolutamide (FDR = 0.29). RNA-seq analysis of tumor samples was performed 72 hours after treatment. Data were compared with the untreated group and hallmark data sets from the Molecular Signatures Database v7.1. **C,** Expression of the differentially expressed genes included in the DNA repair hallmark gene set used in **A**. Red and blue colors indicate higher or lower expression of the selected genes compared with mean expression, respectively, after treatment with 250 kBq/kg PSMA-TTC alone or in combination with 100 mg/kg darolutamide in comparison with untreated control. **D,** γH2AX expression as determined by IHC in untreated ST1273 tumors and in ST1273 tumors treated with 250 kBq/kg PSMA-TTC alone or in combination with 100 mg/kg darolutamide ($n = 3$). IHC analysis was performed 336 hours after treatment. Scale bars indicate 100 μm. **E,** γH2AX expression in tumor tissues shown in **D**, quantified with HSA software. *, $P < 0.05$; **, $P < 0.01$ in comparison with untreated control. FDR, false discovery rate.

The induction of PSMA expression by AR inhibition may also present an opportunity to overcome resistance. Recently, Current and colleagues (46) investigated the efficacy of the radioligand ^{177}Lu-PSMA-617/^{225}Ac-PSMA-617 in RM1 murine prostate cancer cells with varying PSMA expression levels and patterns and found out that low or heterogeneous PSMA expression represented a resistance mechanism to radioligand therapy. In the current study, where VCaP cells with low and heterogenous PSMA expression were inoculated into noncastrated mice, no significant antitumor efficacy was observed upon PSMA-TTC or darolutamide monotherapy, but combination therapy resulted in synergistic antitumor efficacy. This suggests that combination therapy with androgen inhibitors may present a mechanism to overcome treatment resistance caused by low or heterogenous PSMA expression also in the

clinic. Rosar and colleagues demonstrated that, androgen inhibition by enzalutamide increased PSMA expression and PSMA PET tracer uptake in CRPC and that the increase in PSMA expression was even seen in patients with previous enzalutamide treatment failure (45). In line with this, we observed enhanced antitumor activity for the combination treatment with PSMA-TTC and darolutamide in the KUCaP-1 prostate cancer PDX model that has previously been shown to be resistant to enzalutamide therapy (29). Future studies should explore the activity and mode of action of the darolutamide and PSMA-TTC combination treatment in hormone-independent models to clarify whether synergistic antitumor efficacy can be achieved in the absence of direct antiproliferative activity of darolutamide.

Interestingly, in the ST1273 model, with high PSMA level at baseline, no increased PSMA expression was detected upon combination treatment suggesting that PSMA expression may have reached a maximum level. Strong synergistic antitumor efficacy of the PSMA-TTC and darolutamide combination treatment was seen also in this model, and therefore, other potential mechanisms of action were investigated. Our nonbiased RNA-sequencing analysis showed marked induction of DNA repair pathway genes in tumors after PSMA-TTC monotherapy, but not after combination treatment with PSMA-TTC and darolutamide. The combination treatment was also found to induce higher levels of DNA damage compared with PSMA-TTC alone, as detected by the marker γH2AX. Therefore, combining the AR inhibitor darolutamide with PSMA-TTC is suggested to benefit from, not only increased PSMA expression as discussed above, but also reduced DNA repair gene expression, and thereby, increased DNA damage and antitumor efficacy. These findings expand on previous investigations with external beam radiation where AR inhibition has been demonstrated to lead to downregulation of DNA repair genes (47–49), resulting in impaired DNA repair and increased radiosensitivity (48, 50). On the basis of the characteristics of the animal model, efficacy may be driven by different mechanisms of action. Induction of PSMA expression appears to be more relevant in low PSMA-expressing models, while impairment of DNA repair gene induction as a contributor to improved antitumor efficacy is more relevant in high PSMA-expressing models. Taken together, our results demonstrate for the first time that AR inhibition impairs the PSMA-TTC–mediated induction of DNA repair genes. Furthermore, the different nature of DNA damage caused by alpha- and beta-radiotherapy may be important in the context of AR inhibition. This is supported by Goodwin and colleagues (50) indicating a role for androgen inhibition particularly in AR-mediated DSB repair. Alpha-emitters are associated with DBSs, whereas beta-emitters mainly cause single-strand DNA breaks (51). Therefore, the combination of PSMA radionuclide therapy with AR inhibitors may be of particular interest for alpha-emitters.

Darolutamide is an AR inhibitor with a very favorable adverse event profile (52), making it an ideal partner for combination therapy. Furthermore, because darolutamide and PSMA-TTC have very different mechanisms of action, no overlapping toxicities were expected. Indeed, we found the combination treatment to be well tolerated in mice as based on body weight monitoring. As described previously (13), a dose-dependent and reversible reduction of white blood cells was observed in PSMA-TTC–treated mice, and this reduction was not affected by combination treatment with darolutamide. Therefore, this mechanism-based combination treatment potentially presents an effective and safe new treatment option for patients with prostate cancer, and a possibility to widen the therapeutic window of PSMA-TTC treatment. A similar combination effect was observed with enzalutamide, and therefore, the presented data support clinical evaluation of the combination of PSMA-directed radionuclide therapy also with other AR inhibitors.

In summary, PSMA-TTC shows synergistic antitumor efficacy in combination with darolutamide in preclinical prostate cancer xenograft models *in vitro* and *in vivo* with no overlapping toxicities. The combination of PSMA-TTC and darolutamide inhibits tumor growth by a dual mode of action, where darolutamide (i) induces PSMA expression resulting in higher tumor uptake of PSMA-TTC, and consequently, higher antitumor efficacy, and (ii) impairs the PSMA-TTC-mediated induction of DNA repair genes, potentially contributing to increased DNA damage. This may provide new treatment options for patients with prostate cancer independent of their PSMA expression level. A first-in-human trial with PSMA-TTC in patients with mCRPC is currently ongoing (NCT03724747). The data presented provide a strong rationale for further investigation of PSMA-TTC in combination with AR inhibition in patients with advanced prostate cancer.

Authors' Disclosures

S. Hammer reports a patent for Combination of AR antagonists and targeted thorium conjugates, pending. A. Schlicker reports he is an employee and shareholder of Bayer AG. S. Zitzmann-Kolbe reports personal fees from Bayer AG during the conduct of the study. S. Baumgart reports other support from Bayer AG during the conduct of the study and other support from Bayer AG outside the submitted work. U.B. Hagemann reports a patent for Combination of AR antagonists and targeted thorium conjugates, pending. A. Scholz reports other support from Bayer AG and Bayer AD outside the submitted work. B. Haendler reports a patent for Method for determining the response of patients with prostate cancer to treatment with AR antagonists based on gene expression changes or superenhancer protein-binding profiles pending and a patent for Combination of AR antagonists and targeted thorium conjugates, pending and is a full-time employee and shareholder of Bayer AG, which has a commercial interest in the development of drugs for treatment of prostate cancer. P. Lejeune reports a patent for Combination of AR antagonists and targeted thorium conjugates, pending. J. Karlsson reports a patent for Combination of AR antagonists and targeted thorium conjugates, pending. H. Hennekes reports other support from Bayer AG during the conduct of the study and other support from Bayer AG outside the submitted work. C.H. Nielsen reports grants from Bayer AG during the conduct of the study. M.U. Juul reports grants from Bayer AG during the conduct of the study. D. Mumberg reports personal fees from Bayer AG outside the submitted work. C.A. Schatz reports personal fees from Bayer AG outside the submitted work; in addition, C.A. Schatz has a patent for BHC193018, pending. No disclosures were reported by the other authors.

Authors' Contributions

S. Hammer: Conceptualization, data curation, formal analysis, supervision, writing–original draft. **A. Schlicker:** Data curation, software, formal analysis, writing–review and editing. **S. Zitzmann-Kolbe:** Data curation, formal analysis. **S. Baumgart:** Data curation, software, formal analysis. **U.B. Hagemann:** Data curation, formal analysis, writing–review and editing. **A. Scholz:** Data curation, formal analysis, writing–review and editing. **B. Haendler:** Conceptualization, data curation, writing–review and editing. **P. Lejeune:** Conceptualization, data curation, formal analysis, supervision, methodology, writing–review and editing. **J. Karlsson:** Data curation, methodology. **C. Ellingsen:** Data curation, methodology. **H. Hennekes:** Supervision, writing–review and editing. **C.H. Nielsen:** Data curation. **M.U. Juul:** Data curation, formal analysis. **D. Mumberg:** Conceptualization, funding acquisition, writing–review and editing. **C.A. Schatz:** Conceptualization, data curation, formal analysis, supervision, writing–original draft.

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