Darolutamide potentiates the antitumor efficacy of a PSMA-targeted thorium-227 conjugate (PSMA-TTC) by a dual mode-of-action in prostate cancer models

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List of abbreviations: ADT, androgen deprivation therapy; AR, androgen receptor; BID, twice daily; CDX, cell line-derived xenograft; CR, complete response; CRPC, castration-resistant prostate cancer; CSPC, castration-sensitive prostate cancer; DAB, 3,3'-
diaminobenzidine; DMSO, dimethyl sulfoxide; DSB, DNA double strand break; FDR, false discovery rate; GSEA, gene set enrichment analysis; γ-H2AX, phosphorylated histone protein H2AX; HOPO, hydroxypyridinone; HRP, horseradish peroxidase; IHC, immunohistochemistry; mCRPC, metastatic CRPC; mCSPC, metastatic CSPC; IHC, immunohistochemistry; PCa, prostate cancer; PD, progressive disease; PDX, patient-derived xenograft; PET, positron emission tomography; pChk1, phosphorylated Chk1; pChk2, phosphorylated Chk2; PR, partial response; PSMA, prostate specific membrane antigen; once daily; TAT, targeted alpha therapy; T/C, treatment/control; TTC, targeted thorium-227 conjugate; SD, stable disease.

**Key words:** targeted alpha therapy, prostate cancer, PSMA, thorium-227, darolutamide, enzalutamide, androgen receptor
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-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 (1) induces PSMA expression, resulting in increased PSMA-TTC tumor uptake and enhanced efficacy in a model with low baseline PSMA expression, and (2) 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.
ABSTRACT

**Purpose:** Androgen receptor (AR) inhibitors are well-established in the treatment of castration-resistant prostate cancer (CRPC) and have recently shown efficacy also in castration-sensitive prostate cancer (CSPC). 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- 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/Discussion:** 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 radiation therapy, 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) (3-5). CRPC is treated with AR inhibitors, such as competitive AR antagonists or androgen biosynthesis inhibitors, but unfortunately, most patients develop resistance after 18-24 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 3 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 non-metastatic CRPC, and it is currently undergoing clinical investigations in patients in other clinical settings, such as metastatic castration-sensitive prostate cancer (mCSPC) (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 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).
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.
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 (Wuppertal, Germany) and Bayer AS (Oslo, Norway) as described previously (13). Darolutamide was synthesized at Orion Corporation (Espoo, Finland). Enzalutamide, the murine anti-PSMA monoclonal antibody J591 and the synthetic androgen agonist R1881 were synthesized at Bayer AG.

Cancer cell lines and PDX models

VCaP, C4-2 and 22Rv1 prostate cancer cells were obtained from ATCC (Manassas, VA, USA), 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 manufacturer’s protocols at 37ºC in a humidified atmosphere containing 5% CO₂. For the *in vitro* experiments, LNCaP and VCaP cells were supplemented with 0.1 nM of the synthetic androgen agonist R1881. The patient-derived xenograft (PDX) prostate cancer model ST1273 (14) was obtained from South Texas Accelerated Research Therapeutics (San Antonio, TX, USA) 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, USA).

*In vitro* combination assay with PSMA-TTC and darolutamide

The *in vitro* anti-proliferative 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 nM – 37.5 μM). LNCaP cells were treated with fixed-ratio combinations of PSMA-TTC (0.020 – 20 kBq/ml) and darolutamide (36.6 nM – 37.5 μM). 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 (CI) 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 µM darolutamide and 5 kBq/ml PSMA-TTC as mono- 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 (ThermoFischer Scientific) for quantitative RT-PCR (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 to vehicle using the $2^{\Delta\Delta Ct}$ 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 – 5 µM 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 monoclonal antibody 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. Dimethyl sulfoxide (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 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 mM citrate supplemented with 50 mg/ml sucrose, 70 mM NaCl, 0.5 mg/ml para-aminobenzoic acid, 2mM 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 pre-dosed i.v. with 200 μg of an irrelevant mouse antibody (IgG2a, κ isotype, murine myeloma monoclonal UPC10 antibody, Sigma-Aldrich, St. Louis, MO, USA) 16–24 h prior to treatment with PSMA-TTC (17).

In the VCaP efficacy study, male CB17-Scid mice (6 weeks, 22±2 g, Janvier Labs, France) were inoculated subcutaneously with 3 x 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 (i.v.) 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 (BID), p.o.) 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 x 5 x 5 mm 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, i.v.), darolutamide (15, 30 or 100 mg/kg, BIDx21, p.o.) 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, i.v.), darolutamide (100 mg/kg, BIDx21, p.o.) 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, i.v.), enzalutamide (30 mg/kg, once daily (QD)x28, p.o.) or their combinations.

In the KUCaP-1 efficacy study, male CB17-Scid mice (8 weeks, 20 g, Janvier Labs) were implanted subcutaneously with 5 x 5 x 5 mm 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, Q2Wx2, i.v.), darolutamide (200 mg/kg, QD, p.o.) 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 (#6334 for Chk2, #2197 for pChk2, #2348 for pChk1) and OriGene (#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 anti-coagulated 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 \(\sim 1000 \text{mm}^3\). At study termination, the animals were sacrificed by cervical dislocation under \(\text{CO}_2\) 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 criteria (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 h after treatment with 250 kBq/kg PSMA-TTC alone or in combination with darolutamide (100 mg/kg, BID), 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-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) (20). Differential gene expression analysis was performed using the DESeq2 package (version 1.26.0) (21) for the R Project for Statistical Computing (version 3.6.3; https://www.r-project.org/) (22) by comparing mRNA expression to untreated control samples for each treatment. Genes were defined as differentially expressed with absolute
log2 fold-changes >1 and false discovery rates < 0.1. Gene set enrichment analysis (GSEA) was performed using the Fast Gene Set Enrichment Analysis (fgsea) package for R (version 1.10.1) (23) with log2-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 (γ-H2AX) 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, QD, p.o.) for 72, 168 or 336 hours. Sections were fixed at 4°C for 5 min, air-dried, washed with ddH2O and incubated with Dako S2023 Peroxidase-Blocking Solution (Agilent Technologies) at room temperature for 10 min. Next, the sections were washed with Tris-buffered saline and incubated with the mouse anti-phospho-histone H2A.X (Ser139) antibody (1:500; clone JBW301, Merck Millipore, Germany) at room temperature for 60 min, followed by incubation with the horseradish peroxidase (HRP)-labeled anti-mouse polymer (Dako). Immunoreactions were visualized using 3,3’-diaminobenzidine (DAB) as a substrate. Positive γ-H2AX 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 family-wise error rate using either Dunnett’s or Sidak’s 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 standard error as the mean and standard deviation 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’s 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 androgen receptor inhibitors as described previously (26-28). Indeed, the treatment of VCaP cells with darolutamide resulted in an over 10-fold elevated expression of the PSMA gene compared to 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 μM darolutamide upregulated the cell surface PSMA expression in both VCaP and LNCaP cells (Fig. 1E-F) as indicated by 9- and 8-fold increases in comparison to baseline control, respectively. Similar results were obtained with 2.5 μM enzalutamide with 6- and 7-fold 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 (p21Cip1), a gene linking DNA damage to cell cycle arrest, as described previously (13) (Fig. S1A-B). 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

Based on 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 (Fig. S2A). In the VCaP model with low and heterogeneous PSMA expression (Fig. S2A-B), 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-B). However, combination therapy with 150 or 300 kBq/kg PSMA-TTC and darolutamide inhibited tumor growth compared to 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 kBq/kg 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 based on 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 3-fold increase of thorium-227 uptake was observed in VCaP tumors upon PSMA-TTC and darolutamide combination therapy compared to 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, Fig. S3), indicating alpha-particle-induced formation of DNA double strand breaks (DSBs) 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 (Fig. S2A,C), 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, BID) 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 (BID) 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 (BID) darolutamide, respectively (Table 1). In order to limit potential side effects of PSMA-TTC therapy, we explored if 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, BID) 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 (BID) darolutamide showed enhanced antitumor efficacy and a synergistic effect (92.3–100% confidence on day 19, Fig. 3C-D). Furthermore, similar synergistic effects were observed when ST1273 tumors were treated with PSMA-TTC in combination with another AR inhibitor, enzalutamide (Fig. S4). A dose-dependent and, importantly, transient reduction of the white blood cell count was observed in mice treated with PSMA-TTC (Fig. S5). No body weight loss was observed in any of the treatment groups, indicating that the treatments were well-tolerated (Fig. S6A-B).

**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 1/10 mice. Darolutamide monotherapy (200 mg/kg, once daily) was efficacious with a T/C ratio of 0.47 but later disease progression occurred in 10/10 mice (Fig. 4A-B, Supplementary table S1). Compared to 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 4/9 mice and stable disease in 1/9 mice up to 33 days after the start of treatment (Fig. 4A-B, Supplementary table S1). No relevant decreases in body weight compared to 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 h after treatment with 250 kBq/kg PSMA-TTC alone or in combination with 100 mg/kg (BID) darolutamide and compared to 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 (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 false discovery rate (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, 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 Fig. S8). Upon combination treatment with darolutamide, downregulation of DNA repair genes was observed (shown in blue; Fig. 5C and more detailed in 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 h of treatment with 250 kBq/kg PSMA-TTC alone and in combination with 100 mg/kg (BID) darolutamide. Highest γH2AX levels were detected in the combination group 336 h after treatment (Fig. 5D-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 inter- and intra-patient 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 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 non-overlapping 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, i.e. 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 positron emission tomography (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 androgen receptor 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.

The induction of PSMA expression by androgen receptor inhibition may also present an opportunity to overcome resistance. Recently, Current *et al.* (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 present study, where VCaP cells with low and heterogeneous PSMA expression were inoculated into non-castrated 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 heterogeneous PSMA expression also in the clinic. Rosar *et al.* 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 if 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 non-biased 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 to 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). Based on 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 radiation therapy may be important in the context of AR inhibition. This is supported by Goodwin et al. (50) indicating a role for androgen inhibition particularly in AR-mediated DSB repair. Alpha emitters are associated with DSBs, 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, since 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 (1) induces PSMA expression resulting in higher tumor uptake of PSMA-TTC, and consequently, higher antitumor efficacy, and (2) 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.
ACKNOWLEDGMENTS
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FIGURE LEGENDS

Figure 1. Darolutamide and PSMA-TTC combination shows synergistic antitumor efficacy in vitro. A and B, Isobolograms for the in vitro combination effect of PSMA-TTC and darolutamide on the proliferation of (A) VCaP and (B) LNCaP prostate cancer cells. C and D, PSMA (FOLH1) expression in (C) VCaP and (D) LNCaP cells treated with 5 kBq/mL PSMA-TTC and/or 2 μM darolutamide as determined by qRT-PCR 48 h after treatment (n = 2). The expression is presented as fold change compared to untreated cells. E and F, Cell surface PSMA expression as determined by flow cytometry in darolutamide or enzalutamide-treated (E) VCaP and (F) LNCaP cells. DMSO served as a baseline control and is depicted with a dashed line. CI, combination index; DMSO, dimethyl sulfoxide.

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 (i.v., total protein dose 0.14 mg/kg, treatment day indicated with a green arrow) and/or 100 mg/kg darolutamide (BIDx23, p.o, 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 h 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’s or Sidak’s method. *, p<0.05; **, p<0.01; ***, p<0.001 in comparison to vehicle. †, p<0.05; ††, p<0.01; †††, p<0.001 in comparison to the corresponding darolutamide monotherapy. ‡, p<0.01; ‡‡, p<0.001 in comparison to the corresponding PSMA-TTC monotherapy.
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, i.v., treatment day indicated with a green arrow) and/or 15, 30 or 100 mg/kg darolutamide (BIDx21, p.o; 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 (i.v., total protein dose 0.14 mg/kg; treatment day indicated with a green arrow) and/or 100 mg/kg darolutamide (BIDx21, p.o; 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 ≥ 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’s method. **, p<0.01; ***, p<0.001 in comparison to vehicle. #, p<0.05; ##, p<0.01; ###, p<0.001 in comparison to the corresponding darolutamide monotherapy. †, p<0.05; ††, p<0.01; †††, p<0.001 in comparison to the corresponding PSMA-TTC monotherapy.

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 (Q2Wx2, i.v., total protein dose 0.43 mg/kg, treatment days indicated with green arrows), 200 mg/kg darolutamide (QD, p.o., 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’s method. *, p < 0.05; **, p < 0.01; ***, p < 0.001 in comparison to vehicle on day 33; ###, p < 0.001 in comparison to 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 (BID) 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 darolutamide (FDR = 0.29). RNA-seq analysis of tumor samples was performed 72 h after treatment. Data were compared to 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 panel (A). Red and blue colors indicate higher or lower expression of the selected genes compared to mean expression, respectively, after treatment with 250 kBq/kg PSMA-TTC alone or in combination with 100 mg/kg darolutamide in comparison to untreated control. D, γ-H2AX expression as determined by IHC in untreated ST1273 tumor 250 kBq/kg PSMA-TTC alone or in combination with 100 mg/kg darolutamide (n = 3). IHC analysis was performed 336 h after treatment. Scale bars indicate 100 μm. E, γ-H2AX expression in tumor tissues shown in panel (D), quantified with HSA software. *, p<0.05; **, p<0.01 in comparison to untreated control. FDR, false discovery rate.

REFERENCES


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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>T/C ratio on day 33</th>
<th>Response rate on day 100a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CR</td>
</tr>
<tr>
<td>Darolutamide 15 mg/kg&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>0.60</td>
<td>n.a.</td>
</tr>
<tr>
<td>Darolutamide 30 mg/kg&lt;sup&gt;b,d&lt;/sup&gt;</td>
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<td>n.a.</td>
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<tr>
<td>Darolutamide 100 mg/kg&lt;sup&gt;b,e&lt;/sup&gt;</td>
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<td>n.a.</td>
</tr>
<tr>
<td>PSMA-TTC 250 kBq/kg</td>
<td>0.27 **</td>
<td>1/6</td>
</tr>
<tr>
<td>Darolutamide 15 mg/kg + PSMA-TTC 250 kBq/kg</td>
<td>0.01 ***, ###, †,</td>
<td>7/9</td>
</tr>
<tr>
<td>Darolutamide 30 mg/kg + PSMA-TTC 250 kBq/kg</td>
<td>0.01 ***, ###, †,</td>
<td>7/9</td>
</tr>
<tr>
<td>Darolutamide 100 mg/kg + PSMA-TTC 250 kBq/kg</td>
<td>0.01 ***, ###, †,</td>
<td>9/10</td>
</tr>
</tbody>
</table>

n.a., not applicable

<sup>a</sup>Responses were determined according to RECIST criteria.

<sup>b</sup>Treatment period for darolutamide: study days 0 – 20.

<sup>c</sup>Last study day 33

<sup>d</sup>Last study day 36

<sup>e</sup>Last study day 39

**, p<0.01; ***, p<0.001 in comparison to untreated control. ###, p<0.001 in comparison to the corresponding darolutamide monotherapy. †, p<0.05 in comparison to the corresponding PSMA-TTC monotherapy.
**Figure 1**

**A**

![Graph showing EC50 (Darolutamide, M) for VCaP with CI = 0.36.](image)

**B**

![Graph showing EC50 (Darolutamide, M) for LNCaP with CI = 0.47.](image)

**C**

![Bar chart showing PSMA expression (Fold change, mean±SD) for VCaP.](image)

**D**

![Bar chart showing PSMA expression (Fold change, mean±SD) for LNCaP.](image)

**E**

![Graph showing PSMA surface protein (antibodies bound per cell) for VCaP with Darolutamide, Enzalutamide, and DMSO.](image)

**F**

![Graph showing PSMA surface protein (antibodies bound per cell) for LNCaP with Darolutamide, Enzalutamide, and DMSO.](image)
Figure 4

A

B

Tumor volume (mm^3, mean±SEM)

Days after first treatment

0 10 20 30

Tumor weight (mg, mean±SEM)

Vehicle PSMA-TTC (150 kBq/kg) Darolutamide (200 mg/kg) PSMA-TTC (150 kBq/kg, Q2Wx2) + darolutamide (200 mg/kg)

Vehicle PSMA-TTC 150 kBq/kg Darolutamide 200 mg/kg PSMA-TTC + darolutamide

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Darolutamide potentiates the antitumor efficacy of a PSMA-targeted thorium-227 conjugate (PSMA-TTC) by a dual mode-of-action in prostate cancer models

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