SUPPLEMENTARY METHODS for:

Preclinical efficacy of a PSMA-targeted thorium-227 conjugate (PSMA-TTC) - a novel targeted alpha therapy for prostate cancer

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SUPPLEMENTARY METHODS

Synthesis of PSMA-TTC (BAY 2315497)

Thorium-227 was purified from an actinium-227 generator as described previously (1). Briefly, it was purified from its ingrowing daughters by ion exchange chromatography using 2 mL drip columns. An aliquot of the obtained purified thorium-227 HCl solution was dried at 100 °C in a glass vial and used for labeling within 48 hours. The antibody-chelate conjugate solution (BAY 2315493) was incubated with the dried thorium-227 at room temperature for 1 h. The obtained, thorium-227-radiolabeled conjugate solution (BAY 2315497) was sterile-filtered before administration. The percentage of thorium-227 bound to the antibody was analyzed using instant thin layer chromatography (iTLC) using HPGe detector to measure the thorium-227 activity as described previously (2).

In vitro experiments

Internalization of PSMA-TTC (10 nM) into cells was measured after incubation of adherent C4-2 and PC3 PrCa cells in poly-L-lysine (Sigma)-coated 24-well plates coated with PSMA-TTC. After removal of supernatant and washing with PBS, membrane-bound radioactivity was removed by washing twice with glycine-HCl (50 mM, pH 2.8). Internalized activity (% IA) was determined after lysing cells with 0.3 M NaOH and gamma counting analysis of radioactivity in

all fractions using a gamma counter (1470 Wizard automatic gamma counter, Wallac). Results are presented as % IA per 1 x 106 cells.

In vivo studies in subcutaneous xenograft models

The efficacy of PSMA-TTC was studied *in vivo* in cell line-derived and PDX models of prostate cancer (PrCa).

Male CB17-Scid mice (20 g, 9 weeks, Janvier Labs, Le Genest-Saint-Isle, France) were inoculated subcutaneously with 5 x 10^6 LNCaP human PrCa cells (n=10 mice/group). Three days prior to inoculation, the mice were supplemented with a testosterone pellet (12.5 mg, 4 mm, release rate >15 μg/day for 90 days, Bayer AG). The mice were subsequently injected i.v. with vehicle (isotonic saline; 0.9% NaCl, Baxter, Deerfield, IL, USA) or PSMA-TTC (single dose, 75 or 150 or 300 kBq/kg; or Q2Wx2, 150 kBq/kg; or QWx4, 75 kBq/kg), radiolabeled isotype control (300 kBq/kg), or non-radiolabeled PSMA antibody-chelator conjugate at a total antibody dose of 0.43 mg/kg on day 19 (average tumor size 177 mm^3).

Male Athymic Nude mice (18–21 g, 4-6 weeks, Envigo, Huntington, UK) were inoculated subcutaneously with 5 x 10^6 MDA-PCa-2b human PrCa cells (n=10 mice/group). The mice were supplemented with testosterone (4 mg/L, Sigma-Aldrich) in the drinking water. In addition, 42 mice (n=3-6/group per time point) were similarly inoculated for a separate pharmacokinetic experiment. In the efficacy study, the mice were subsequently injected intravenously (i.v.) with

vehicle (30 mM citrate, 70 mM NaCl, 0.5 mg/mL PABA, 2 mM EDTA, pH 5.5), PSMA-TTC (100, 250, or 500 kBq/kg), radiolabeled isotype control (250 kBq/kg), or non-radiolabeled PSMA antibody-chelator conjugate on day 61 (average tumor size 62 mm^3). In the pharmacokinetic experiment, the mice were dosed with PSMA-TTC or radiolabeled isotype control (both at 500 kBq/kg). The tumors, blood, spleen, kidney, liver, muscle and femurs were harvested and analyzed for remaining thorium-227, 0.5 h, 2 h, 5 h, 8 h, 24 h, 3 d, 7 d, 14 d, and 21 d after dosing.

Male NMRI Nude mice (30 g, 5–7 weeks, Taconic, Cologne, Germany) were inoculated subcutaneously with 1 x 10^6 C4-2 human PrCa cells (n=10 mice/group). The mice were subsequently injected i.v. with vehicle (30 mM citrate, 70 mM NaCl, 0.5 mg/mL PABA, 2 mM EDTA, pH 5.5), PSMA-TTC (100, 250, and 500 kBq/kg), radiolabeled isotype control (250 kBq/kg), or non-radiolabeled PSMA antibody-chelator conjugate on day 9 (average tumor size 103.3 mm^3).

Male Athymic Nude mice (24.3–34.9 g, 7–9 weeks, Envigo) were inoculated subcutaneously with 3 x 10^6 22Rv1 human PrCa cells (n=10 mice/group). In addition, mice were similarly inoculated for a separate pharmacokinetic experiment (n=3 mice/group for each time point). For the efficacy study, the mice were subsequently injected i.v. with vehicle (isotonic saline; 0.9% NaCl, Baxter), PSMA-TTC (100, 250, or 500 kBq/kg), radiolabeled isotype control (250 kBq/kg), or non-radiolabeled PSMA antibody-chelator conjugate on day 13 (average tumor size 83.6 mm^3). In the pharmacokinetic experiment, the mice

were dosed with a single dose of vehicle, PSMA-TTC (500 kBq/kg, at a protein dose of 0.14, or 0.75 mg/kg) or a radiolabeled isotype control (500 kBq/kg, at a protein dose of 0.14 mg/kg) on day 25 (average tumor size 314.6 mm^3). Subsequently, the tumors, blood, lungs, kidneys, livers, spleens, left tibiae, and muscles of the mice were harvested and analyzed for remaining thorium-227 0.5 h, 2 h, 5 h, 8 h, 24 h, 3 d, 7 d, 14 d, and 21 d after dosing.

To further study the effect of the total antibody dose of PSMA-TTC on its antitumor efficacy, male Athymic Nude mice (16.4–25.5 g, 5 weeks, Envigo) were inoculated subcutaneously with 3×10^6 22Rv1 PrCa cells (n=10 mice/group). The mice were subsequently injected i.v. with vehicle (isotonic saline; 0.9% NaCl, Baxter), PSMA-TTC (500 kBq/kg), radiolabeled isotype control (250 kBq/kg), or non-radiolabeled PSMA antibody-chelator conjugate on day 17 (average tumor size 100 mm^3). The TTCs were administered at three different total antibody doses: 0.14, 0.75, or 5 mg/kg. Tumors were collected at the end of the study. In a pharmacokinetic experiment, the animals bearing 22Rv1 tumors were dosed with a single dose of PSMA-TTC (500 kBq/kg, at a protein dose of 0.14, or 5 mg/kg) on day 15 at an average tumor size of 200 mm^3. Subsequently, tumors and blood were harvested and analyzed for remaining thorium-227 24, 72, 168, 336, and 504 h after dosing.

To further explore the potential effect of the total antibody dose on the antitumor efficacy of PSMA-TTC, male NMRI Nude mice (31.3–37.6 g, 6 weeks, Janvier Labs) were supplemented with a testosterone pellet (12.5 mg, 4 mm, release

rate >15 μg/day for 90 days, Bayer AG) three days prior to a subcutaneous inoculation with 2×10^6 22Rv1 PrCa cells (n=10 mice/group). Mice were subsequently injected i.v. with vehicle (isotonic saline; 0.9% NaCl, Baxter) or PSMA-TTC (500 kBq/kg at total antibody doses of 0.43 and 1.5 mg/kg), on day 12 (average tumor size 122 mm^3).

Female NMRI Nude mice (23.9–33.7 g, 6–8 weeks, Janvier Labs) supplemented with testosterone (Testosterone MedRod 100 μg/day, PreclinApps, Raisio, Finland) were implanted subcutaneously with 5 x 5 x 5 mm ST1273 human PrCa tumor fragments (n=10 mice/group). In addition, mice were similarly inoculated for a separate pharmacokinetic experiment (n=3 mice/group for each time point). For the efficacy study, the mice were subsequently injected i.v. with vehicle (30 mM citrate, 70 mM NaCl, 0.5 mg/mL PABA, 2 mM EDTA, pH 5.5), PSMA-TTC (125, 250, or 500 kBq/kg), or radiolabeled isotype control (250 kBq/kg) on day 28 (average tumor size 276 mm^3). Blood samples were collected one day before dosing and every other week after dosing for subsequent hematology analyses using ProCyte Dx Hematology Analyzer (Idexx Laboratories, Westbrook, ME, USA). In the pharmacokinetic experiment, the mice were dosed with a single dose of vehicle, PSMA-TTC or a radiolabeled isotype control (both 500 kBq/kg, at a total antibody dose of 0.14 mg/kg) on day 28 (average tumor size 176 mm^3). Subsequently, the tumors, blood, livers, spleens, and femurs of the mice were harvested and analyzed for remaining thorium-227 24 h, 7 d, 14 d, and 21 d after dosing.

Male CB17-Scid mice (20 g, 5-6 weeks, Janvier Labs) were implanted subcutaneously with 5 x 5 x 5 mm KUCaP-1 human PrCa tumor fragments (n=10 mice/group). The mice were subsequently injected i.v. with vehicle (isotonic saline; 0.9% NaCl, Baxter), PSMA-TTC (75, 150, or 300 kBq/kg), or radiolabeled isotype control (300 kBq/kg) on day 23 (average tumor size 150 mm^3).

Male CB17-Scid mice (25 g, 6 weeks, Janvier Labs) were implanted subcutaneously with 5 x 5 x 5 mm LuCaP 86.2 human PrCa tumor fragments (n=10 mice/group). The mice were subsequently treated with vehicle (PEG400/propylene glycol/5% glucose 50/30/20 v/v/v p.o), PSMA-TTC (150 or 300 kBq/kg, i.v.), or radiolabeled isotype control (300 kBq/kg, i.v.) on day 43 (average tumor size 200 mm^3).

In vivo intratibial tumor growth model

LNCaP-luc human PrCa cells (2 x 10^6) were inoculated into the intratibial bone marrow cavity of male NOD.scid mice (19–26 g, 5–6 weeks, Envigo) (n=10-11 mice/group). LNCaP cells secrete PSA and are known to form osteoblastic and mixed lesions when inoculated to the bone marrow cavity (3). To monitor the intratibial tumor growth, bioluminescence imaging (BLI) measurements were performed using the IVIS Lumina imaging system (PerkinElmer, Waltham, MA, USA) at inoculation and four days thereafter. The mice were subsequently injected i.v. with vehicle (30 mM citrate, 70 mM NaCl,

0.5 mg/mL PABA, 2 mM EDTA, pH 5.5) or PSMA-TTC (100 or 200 kBq/kg) on day 6 when BLI signals were detectable and available for use in allocation of mice into treatment groups. Blood samples were collected from vena saphena before cell inoculation, before dosing and once every two weeks thereafter. The development of tumor-induced osteoblastic, osteolytic, and mixed lesions in bone was determined by X-ray imaging and micro-computed tomography (micro-CT) as described below.

Biodistribution studies

The *ex vivo* samples were analyzed for remaining radioactivity using a high-purity germanium detector (HPGe) linked to an autosampler (Gamma Data). To identify thorium-227 and radium-223, the GammaVision software and Npp32 analysis engine (Reg. Guide 4.16 detection limit method) were used. Thorium-227 counts were corrected to the time of injection and expressed as percentage (%) of injected dose of thorium-227 per gram (% ID/g).

X-ray and micro-computed tomography analyses

The development of tumor-induced osteoblastic, osteolytic, and mixed lesions in bone was monitored by X-ray imaging starting on study day 11 and once every two weeks thereafter. X-ray images were taken with the Faxitron Specimen Radiographic System MX-20 D12 (Faxitron Corp., Wheeling, IL, USA) using Faxitron Dicom 3.0 software. At least one X-ray image (both hind

limbs) per animal was taken on each time point (31 kV, 10 seconds, magnification 2x). Healthy and tumor-bearing tibiae from two animals of each group were selected based on X-ray data to create representative 3D constructs using micro-computed tomography (micro-CT). At sacrifice, healthy and tumor-bearing tibiae were collected, weighed, and fixed in 4% PFA (paraformaldehyde) and stored in 70% ethanol and measured using SkyScan 1072 ex vivo microCT (Bruker microCT, Kontich, Belgium) for 3D construction of tibiae (University of Eastern Finland, SIB Labs, Kuopio, Finland).

Immunohistochemistry

To determine PSMA expression in xenograft tumor tissues, immunohistochemistry (IHC) experiments were performed on 3-µm thick paraffin sections using the peroxidase-conjugated avidin-biotin method. The antibody used was monoclonal mouse anti-human prostate-specific membrane antigen (PSMA), clone 3E6 (M3620, Dako Denmark, Glostrup, Denmark) as a 1:1000 dilution in antibody diluent (DAKO S2022, Dako Denmark). In brief, sections were deparaffinized in xylene and rehydrated through graded ethanol at room temperature. Incubation with the primary antibody was performed at room temperature for 120 min. After washing, the sections were incubated with the DAKO Envision secondary system (DAKO K4006, Dako Denmark). Immunoreactions were visualized using 3,3'-diaminobenzidine (DAB) as a substrate. Cell pellets from LNCaP cells (treated similarly to the xenograft samples) were used as a positive control.

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