SUPPORTING METHODS for:

**Mesothelin-targeted thorium-227 conjugate (MSLN-TTC): Preclinical evaluation of a new targeted alpha therapy in mesothelin-positive cancers**

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**SUPPORTING METHODS**

**Synthesis and characterization of BAY 2287411**

Thorium-227 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 ºC in a glass vial and used for labeling within 48 hours. The conjugate BAY 2287409 solution was incubated with the dried thorium-227 at room temperature for 1 h. The obtained, thorium-227-radiolabeled conjugate solution (BAY 2287411) 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.

Dimerization and aggregation of BAY 2287411 was analyzed over the course of 48 hours by SEC-HPLC using a Tosoh TSK SW3000 4.6x300 mm column and 0.3 M NaCl, 0.2 M NH4Ac, 10% (v/v) DMSO and 2 mM DTPA as mobile phase. The recorded monomer peak area of the radio- and the UV signal (280 and 335 nm) were integrated and expressed in % of the total high molecular peak area.

The immunoreactive fraction was determined by using an anti-idiotype antibody to BAY 86-1903 mimicking the epitope of MSLN, which was coupled to magnetic beads. The radiolabeled fraction within BAY 2287411 was captured upon binding to the beads and the percentage of radiolabeled BAY 2287411 bound to antigen was determined.

The binding affinity of BAY 2287411 to recombinant human mesothelin was determined by an ELISA assay. Recombinant human mesothelin was coated to ELISA plates (NUNC; 1 µg/mL). Non-coated surfaces were subsequently blocked using 3% (w/v) bovine serum albumin. BAY 86-1903, BAY 2287409, BAY 2287411 and a non-binding isotype control IgG1 molecule were titrated in a three-fold serial dilution, starting at 3.3 µg/mL. Bound antibodies were detected using horseradish peroxidase coupled anti-human IgG (goat, Southern Biotech) and visualized using peroxidase substrate ABTS, following read-out at 405 nm. For flow cytometry analysis, cell lines were seeded in V-shaped 96-well plates and incubated with a serial dilution of BAY 86-1903 and BAY 2287409, and non-binding isotype control IgG1 starting at 100 µg/mL. Antibodies were detected using a phycoerythrin (PE)-coupled anti-human antibody (Biolegend) and median fluorescence intensities were recorded using an Easy Cyte (8HT, Merck Millipore). Data were evaluated using FlowJo software, visualized using GraphPad Prism software (version 7) and half-effective binding concentrations (EC50) were calculated. The beads of the Quantibrite kit were gated from the forward/side scatter plot according to manufacturer’s instructions. Each individual peak recorded for each bead concentration was gated and the log(median fluorescence intensity of each peak) was plotted against the log(molecules per bead). A linear regression curve fit was used to extrapolate the number of PE molecules from the MFI obtained for each respective cell line where BAY 2287409 was applied at the highest tested concentration and the number of antibodies bound per cell was calculated.

***In vitro* cytotoxicity and mode-of-action experiments**

For determination of *in vitro* cytotoxicity, cells were harvested from culture and seeded into 96-well plates (2000 cells/well) on day -1. On day 0, BAY 2287411 as well as a respective isotype control chelator-conjugate were radiolabeled at specific activities of 10 and 40 MBq/mg and titrated on the cells in a 3-fold serial dilution, starting at a total radioactivity of 20 kBq/mL. Cells were incubated for 5 days at 37°C with 5% CO2 and cell viability was measured following the protocol of the CellTiter-Glo® Kit (Promega, USA). Luminescence was determined on Wallac EnVision™ plate reader and the percent of viability was calculated from raw data using the following formula: viability (%) = (luminescence in well)/(average luminescence in control wells)×100%. GraphPad Prism software was used to calculate the half-maximum inhibitory concentrations (IC50). The ratio of the IC50 values between the radiolabeled isotype control and BAY 22787411 were determined for each cell line, and a calculated value ≥1 accounts for specificity.

The proposed mode-of-action of BAY 2287411 was studied in OVCAR-3 and HT29-MSLN cells. Cells were cultured and treated with BAY 2287411 at various concentrations in 6-well plates. After three days, cells were harvested and analyzed for danger-associated molecular pattern molecules (DAMPs) using calreticulin, HSP70, HSP90, and HMGB1 antibodies (all Bioss, USA) upon incubation for 1 h before an easyCyte™ (8HT; Merck Millipore) measurement.

For the determination of DNA double-strand breaks and the cell cycle analysis, the cells were fixed using 70% ice-chilled EtOH. Cells were incubated in the presence of PE-labeled anti-phospho-histone H2A.X (Ser139) antibody (Biolegend) and Alexa-Fluor® 488 anti-cleaved caspase-3 (Asp175) antibody (Cell Signaling) for 1 h simultaneously and subsequently analyzed using easyCyte™. The level of cleaved caspase3/phospho-histone H2A.X positive cells was determined.

In parallel, cells were stained for 10 min with PI RNAse (Thermo Fisher) and the cell cycle phase was determined using easyCyte™ by recording the fluorescence signal generated through the RED-B filter in a linear scale.

For determination of apoptosis, the induction of cleaved caspase-3 was evaluated. Cells (900 cells/well) were incubated in the presence or absence of the pan-caspase inhibitor zVAD-FMK (Promega) at a final concentration of 20 µM. Cells were treated with BAY 2287411 or a respective isotype control chelator-conjugate at decreasing concentrations starting at 20 kBq/mL, and incubated for 4 days at 37°C with 5% CO2. Induction of cleaved caspase-3 and cell viability were measured with the Cleaved Caspase-3/7-Glo® Kit (Promega) and the CellTiter-Glo® Kit (Promega), respectively. Luminescence was determined with PHERAstar FSX (BMG LABTECH) plate reader and the percent of viability was normalized to controls.

Induction of reactive oxygen species (ROS) was determined with the ROS-Glo-H2O2 kit (Promega). Cells (900 cells/well) were treated with BAY 2287411 or a respective isotype control chelator-conjugate at decreasing concentrations starting at 20 kBq/mL, and incubated for 5 days at 37°C with 5% CO2. Luminescence was recorded with a PHERAstar FSX (BMG LABTECH) plate reader. The increase in ROS was calculated by normalizing values to cells grown in presence of medium.

Induction of necrosis was determined with the RealTime-Glo Necrosis assay kit (Promega). HT29-MSLN cells (900 cells/well) were incubated in presence or absence of necrostatin-1 inhibitor (Sigma Aldrich) at a final concentration of 20 µM (or DMSO as control). Cells were treated with BAY 2287411 or a respective isotype control chelator-conjugate at decreasing concentrations starting at 20 kBq/mL. Non-radiolabeled MSLN antibody-chelator conjugate and cells grown in medium were included as controls. Cells were incubated for 5 days at 37°C with 5% CO2 and fluorescence (485nmEx/520–30nmEm) was measured with a PHERAstar FSX (BMH LABTECH) plate reader. The increase in necrosis was normalized to cells grown in presence of medium.

**Immunofluorescence on cells**

For *in vitro* immunofluorescence staining, HT29-MSLN and OVCAR-3 cells were seeded at densities of 20,000 and 10,000 cells/well in LabTek II chamber slides (Nunc). Cells were incubated in presence of 5 kBq/mL BAY 2287411 or non-radiolabeled BAY 2287409 for 4 days. The medium was removed and cells were washed with PBS (Ca2+/Mg2+) and subsequently fixed using PFA (3.7 % (v/v)). Cells were permeabilized using PBS, supplemented with Triton X-100 (0.25 % (v/v)), washed and blocked using Odyssee blocking solution (Licor). Cells were incubated with Anti-Cytochrome C antibody (mouse, clone 37BA11, Abcam) and anti-γH2AX antibody (rabbit, 2OE3, Cell Signaling) for 1 h and subsequently washed using PBS-Tween (0.05 %(v/v)). Samples were incubated with Alexa Fluor goat anti-mouse IgG Plus 488 (Invitrogen) and Alexa Fluor goat anti-rabbit IgG Plus 647 (Invitrogen) antibodies for 1 h and washed using PBS-Tween (0.05 %(v/v)). Cells were further incubated in ProLong™ Diamond Antifade Mountant with DAPI (Invitrogen). Images were obtained with Axio Scan-Z1 microscope (Zeiss) using the respective fluorescence filters. In parallel, an additional set of cells, seeded and treated under the same conditions, were stained for alive cells, apoptosis and necrosis using the Apoptosis/Necrosis Detection kit (Abcam).

***In vivo* antitumor efficacy studies**

Female BomTac:NMRI-*Foxn1nu* nude mice (20–25 g, 6–8 weeks, Taconic-Europe, Germany) were inoculated subcutaneously with 2 x 106 BxPC-3 human pancreatic cancer 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, BAY 2287411 (100, 250 or 500 kBq/kg), or BAY 2473626 (250 kBq/kg), at a total antibody dose of 0.14 mg/kg at an average tumor size 60 mm3. In the pharmacokinetic experiment, the mice were dosed with a single dose of vehicle, BAY 2287411 (500 kBq/kg), or BAY 2473626 (250 kBq/kg), both at a total antibody dose of 0.14 mg/kg at an average tumor size of 200–300 mm3. Subsequently, the tumors, blood, and intact femur were harvested from the mice and analyzed for remaining thorium-227 at time points 0.5 h, 2 h, 5 h, 24 h, 3 d, 7 d, and 14 d after dosing.

Female BALB/c-*nu* nude mice (20 g, 7–8 weeks, Janvier Labs, France) were inoculated subcutaneously with 1 x 106 Capan-2 human pancreatic cancer cells (n=10 mice/group). In addition, mice were similarly inoculated with 2.5 x 106 Capan-2 cells for a separate pharmacokinetic experiment (n=3 mice/group for each time point). For the efficacy study, the mice were subsequently injected intravenously (i.v.) with a single dose of vehicle, BAY 2287411 (125, 250 or 500 kBq/kg), BAY 2473626 (250 kBq/kg), or 4 x 125 kBq/kg (QW) or 2 x 250 kBq/kg (QW) of BAY 2287411 on day 14 (average tumor size 86 mm3). Repeated dosings were performed at weekly intervals. Blood samples were collected before dosing and at two-week intervals up to and including termination and analyzed for platelet, red blood cell and white blood cell counts. In the pharmacokinetic experiment, the mice were dosed with a single dose of vehicle, BAY 2287411, or a BAY 2673626 (both at 500 kBq/kg, at a total antibody dose of 0.14 mg/kg) on day 16 (average tumor size 86 mm3). Subsequently, tumors and blood were harvested and analyzed for remaining thorium-227 at time points 24 h, 3 d, 7 d, 14 d, and 21 d after last dosing.

Female BomTac:NMRI-*Fox1nu* nude mice (20–25 g, 6–8 weeks, Taconic-Europe, Denmark) were inoculated subcutaneously with 1 x 106 HT29-MSLN human colorectal cancer cells (n=12 mice/group). In addition, mice were similarly inoculated with 2.5 x 106 HT29-MSLN cells for a separate pharmacokinetic experiment (n=6 mice/group for each time point). For the efficacy study, the mice were subsequently injected i.v. with vehicle, BAY 2287411 (100, 250 or 500 kBq/kg), BAY 2473626 (100, 250 or 500 kBq/kg), or BAY 2287409 on day 5 (average tumor size 100–250 mm3), all at a total antibody dose of 0.14 mg/kg. In the pharmacokinetic experiment, the mice were dosed with a single dose of vehicle, BAY 2287411 (500 kBq/kg, at a total antibody doses of 0.029, 0.14 or 0.75 mg/kg) or BAY 2473626 (500 kBq/kg, at a total antibody dose of 0.14 mg/kg) on day 5 (average tumor size 100–250 mm3). Subsequently, the tumors, blood, lungs, kidneys, liver, spleen, intestines, femur and muscle, heart, and tail were harvested and analyzed for remaining thorium-227 at time points up to 21 d after dosing.

To assess the crossfire effect of BAY 2287411, i.e., the cells inaccessible to the targeting antibody being nevertheless eradicated by the ionizing radiation even at a distance, female BomTac:NMRI-*Foxn1nu* nude mice (20–25 g, 6-8 weeks, Taconic-Europe, Germany) were inoculated subcutaneously with cell mixtures of MSLN-transfected HT29-MSLN colorectal cancer cells and the parental, non-transfected MSLN-negative HT29 cells at various different ratios, 1 x 106 cells in total (n=10 mice/group). The mice were subsequently injected i.v. with a single dose of vehicle, or BAY 2287411 (500 kBq/kg; total antibody dose of 0.14 mg/kg) on day 10 (average tumor size 44 mm3).

The disseminated model using NCI-H226-*luc* (NMI, Tuebingen/Germany) human lung cancer cells was used to mimic metastasized (or disseminated) lung cancer in patients. Female HsdCpb athymic Nude-*Foxn1nu* nude mice (18–21 g, 6 weeks, Harlan, The Netherlands) were inoculated i.v. with 2.5 x 105 NCI-H226-*luc* lung cancer cells (n=10 mice/group). The mice were subsequently injected intravenously (i.v.) with vehicle, BAY 2287411 (100, 250 or 500 kBq/kg), BAY 2473626 (250 kBq/kg), or BAY 2287409 on day 8 (average tumor size 2.45 x 10-8 p/s/m2/sr, as measured by BLI), all at total antibody doses of 0.14 mg/kg. To evaluate tumor burden, animals were subjected to weekly BLI measurements using PerkinElmer Quantum imaging system and Living Image software.

Female BomTac:NMRI-*Fox1nu* nude mice (20–25 g, 6–8 weeks, Taconic-Europe, Germany) were inoculated subcutaneously with 3 x 106 OVCAR-3 human ovarian cancer cells (n=10 mice/group). In addition, mice were similarly inoculated (n=3 mice/group for each time point) for a separate pharmacokinetic experiment. For the efficacy study, the mice were subsequently injected i.v. with a single dose of vehicle, BAY 2287411 (250 kBq/kg, at total antibody doses of 0.14 or 0.75 mg/kg), or BAY 2473626 (250 kBq/kg, at a total antibody dose of 0.75 mg/kg) at an average tumor size of 36 mm3. For the pharmacokinetic experiment, the mice were subsequently injected i.v. with a single dose of vehicle, BAY 2287411 (500 kBq/kg, at total antibody doses of 0.14 or 0.75 mg/kg), or BAY 2473626 (250 kBq/kg, at a total antibody dose of 0.75 mg/kg) at an average tumor size of 200–300 mm3). The tumors and blood were harvested and analyzed for remaining thorium-227 at time points 24 h, 3 d, 7 d and 21 d after dosing.

Female RjOrI:NMRI-*Foxn1nu/Foxn1nu* nude mice (24–30 g, 6 weeks, Janvier Labs, France) were implanted subcutaneously with patient-derived ST103 human ovarian tumor fragments (n=10 mice/group), provided by START (South Texas Accelerated Research Technology, Houston/USA). In addition, mice were similarly implanted 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 a single dose of vehicle, BAY 2287411 (125, 250 or 500 kBq/kg), BAY 2473626 (250 kBq/kg), or BAY 2287409, or 2 x 250 kBq/kg or 4 x 125 kBq/kg BAY 2287411, all at a total antibody dose of 0.14 mg/kg at an average tumor size of 213 mm3. Repeated dosings were performed at weekly intervals. In the pharmacokinetic experiment, the mice were dosed with a single dose of BAY 2287411 (500 kBq/kg) or BAY 2473626 (500 kBq/kg), both at a total antibody dose of 0.14 mg/kg at an average tumor size of 200-300 mm3). Subsequently, tumors, blood, heart, liver, spleen, and femurs were harvested and analyzed for remaining thorium-227 at time points 0.5 h, 24 h, 3 d, 7 d, 14 d, 21 d, and 28 d after last dosing.

Female RjOrI:NMRI-*Fox1nu/Fox1nu* nude mice (24-30 g, 6 weeks, Janvier Labs, France) were implanted subcutaneously with patient-derived ST2185B TNBC fragments (n=10 mice/group), provided by START. 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 a single dose of vehicle, BAY 2287411 (125, 250 or 500 kBq/kg), BAY 2473626 (250 kBq/kg), or BAY 2287409, or 2 x 250 kBq/kg or 4 x 125 kBq/kg BAY 2287411, all at a total antibody dose of 0.14 mg/kg at an average tumor size of 96 mm3. Repeated dosings were performed at weekly intervals. Blood samples were collected before dosing and at two-week intervals and at sacrifice. In the pharmacokinetic experiment, the mice were dosed with a single i.v. dose of BAY 2287411 (500 kBq/kg) or BAY 2473626 (500 kBq/kg), both at a total antibody dose of 0.14 mg/kg at an average tumor size of 200–300 mm3. Subsequently, tumors and blood were harvested and analyzed for remaining thorium-227 at time points 24 h, 3 d, 14 d, and 21 d after last dosing.

**Measurement of accumulated radioactivity in biodistribution studies**

The accumulated radioactivity was counted using a high-purity germanium detector (HPGe) linked to an autosampler (Gamma Data). To identify thorium-227, the GammaVision software and Npp32 analysis engine (Reg. Guide 4.16 detection limit method) were used. For thorium-227 measurement, the 235.96 keV (abundance 12.90%), 256.23 keV (abundance 7.00%), 329.85 keV (2.90% abundance), 286.09 keV (abundance 1.74%), 304.50 keV (abundance 1.15%), 334.37 keV (abundance 1.14%), and 299.98 keV (abundance 2.21%) gamma peaks 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).

**Immunohistochemistry on tumor sections**

For generation of formalin-fixed, paraffin-embedded (FFPE) samples of cell line and patient-derived xenograft models, tumor sections were fixed in 10% buffered formalin for 24 h following dehydration and embedded in paraffin using a Leica ASP200S tissue processor. Automated IHC was performed on 4 µm histological FFPE sections on a Ventana Discovery autostainer using DAB detection chemistry with anti-rabbit-HQ and anti-MSLN antibody (clone SP74, Spring Biosciences) at 0.25 µg/mL antibody concentration.

Percentage of tumor cells staining positive for MSLN at each intensity level (0, 1, 2, or 3) was calculated and an H-score was calculated as described previously (1).

Similarly, frozen tumor tissue sections isolated from the Capan-2 xenograft model were prepared from animals treated with vehicle, radiolabeled isotype control (250 kBq/kg), BAY 2287411 (a single dose of 500 kBq/kg, or 4x125 kBq/kg, Q7D) and stained using an anti-MSLN antibody (SP74, Spring Biosciences).

Paraffin-embedded sections isolated from the HT29-MSLN xenograft model were prepared from animals treated with BAY 2287411 at 100, 250 and 500 kBq/kg. Sections were fixed at 4°C for 5 min, air-dried and washed with H2Odd before incubation (10 min, room temperature) with DAKO blocking solution. Sections were washed subsequently with Tris buffer saline and incubated with the γH2AX antibody (Millipore) or cleaved caspase-3 antibody (Cell Signaling) for 60 min at room temperature. After respective washing steps, the primary antibodies were detected using HRP-labeled-anti-mouse polymer (Dako) and subsequently visualized using DAB solution. Positive γH2AX signals were counted and plotted in GraphPad PRISM software.

**PK/PD modeling**

A mechanistic PK/PD model to describe PK and PD of non-radiolabeled antibody-chelator conjugate, thorium-227-related radioactivity and receptor turnover in tumor *in vivo* was developed as follows:

After i.v. administration of BAY 2287411, the antibody-chelator conjugate (radiolabeled and non-radiolabeled) is eliminated from the central compartment according to the clearance (CL) and is reversibly distributed to the peripheral compartment and into the tumor.

The antibody-chelator conjugate in the tumor reversibly binds to the antigen MSLN expressed on tumor cells according to its association rate constant (kon) and the dissociation rate constant of the antibody-chelator conjugate-MSLN complex (koff). The respective values for kon and koff were set to the *in vitro* determined values (kon=0.36 nM-1·h-1, koff= 5.15 h-1;Biacore), corresponding to a dissociation constant (KD = koff/kon) of the conjugate-target complex of ~14 nM. Synthesis and degradation rate of the receptor (ksyn, kdeg) are also accounted for in the model and were determined by fitting. After internalization, the antibody-chelator conjugate-antigen complex is degraded by proteolytic cleavage and thorium-227-bearing metabolites are formed upon decay. Due to their poor permeability, the elimination of the thorium-227-bearing metabolites (e.g. antibody-chelator conjugate degradation products) from the tumor occurs very slowly and a redistribution of systemically available thorium-227-bearing metabolites to the tumor can be neglected.

Upon radioactive decay of thorium-227 (thorium-227 antibody-chelator conjugate, thorium-227-containing metabolites), radium-223 is formed. Due to its very poor affinity, radium-223 is immediately released from the chelator and the corresponding non-radiolabeled antibody-chelator conjugate and metabolites are formed.

The model was applied to describe the PK of BAY 2287411 in tumor bearing mice after i.v. administration of different total antibody doses. Target densities in the various xenograft models were set to *in vitro* determined values (4,200–242,413 receptors/cell). All c/t-profiles in blood and tumor were fitted simultaneously to the mechanistic PK model described above. The percentage of target engagement and cumulative hits/tumor cell were then calculated based on the estimated PK parameters.

The estimated PK parameters were used for fitting the tumor growth time course in mice relating efficacy to tumor thorium-227 activity using a transit compartment model as described in literature (2).

A schematic illustration of the PK/PD model is shown in **Fig. 4A**. The simulation of c/t-profiles and cumulative hits was done as follows. The number of receptors per cell was varied between 5,000 and 800,000. Assuming 40,000,000 tumor cells/g tumor, this corresponds to a baseline antigen density of ~3.3 nM and 531 nM, respectively. The total antibody dose was varied between 0.03 to 0.75 mg/kg.

The PK/PD analysis was carried out using Phoenix® (version 6.4, Pharsight, Certara, L.P., Princeton, NJ, USA). Data fitting was performed on the mean values of all measured data applying the naïve pooled algorithm.

**REFERENCES FOR SUPPLEMENTARY MATERIAL**

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