SUPPLEMENTARY METHODS

ImmunoPET of Ovarian and Pancreatic Cancer with AR9.6, a Novel MUC16-Targeted Therapeutic Antibody

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EXPERIMENTAL METHODS

Cell Culture

All cells used in this study were grown as adherent monolayers in a 37 °C incubator providing humidified air with 5% CO_2. OVCAR3 cells were cultured using Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with heat inactivated fetal calf serum (FCS) (20% v/v, GIBCO, Life Technologies), 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/L glucose, 1.5 g/L sodium bicarbonate, 0.01 mg/mL bovine insulin (Gemini Bio-Products, 700-112P), 100 units/mL penicillin and 100 µg/mL streptomycin (Pen-Strep). SKOV3 cells were cultured in RPMI McCoy's 5A Medium, supplemented with 1.5 mM L-glutamine, 100 units/mL penicillin G and 100 µg/mL streptomycin and 10% FCS. The cell lines were sub-cultured by splitting a T-150 flask (1:4) once per week for OVCAR3 cells and 1:6 for SKOV3 cells using 0.25% trypsin/0.53 mM EDTA in Hank's Buffered Salt Solution without calcium and magnesium. Capan-2 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing high glucose and supplemented with 20% FCS and 100 µg/mL Pen-Strep. BxPC-3 cells were cultured in RPMI 1640 media containing supplemented with 10% FCS, non-essential amino acids, 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/L glucose, and 1.5 g/L sodium bicarbonate and 100 µg/mL Pen-Strep. MIAPaCa-2 cells were cultured in DMEM containing high glucose and supplemented with 2.5% horse serum, 10% FCS, 4 mM L-glutamine, 4.5 g/L glucose, 1 mM sodium pyruvate, 1.5 g/L sodium bicarbonate and 100 µg/mL Pen-Strep. S2-028 and T3M-4 cells were cultured in RPMI 1640 medium supplemented with 10% FCS and 100 µg/mL Pen-Strep.

Authentication of Cell Lines

Pancreatic cancer cell line S2-028 (SUIT-2) was provided to the Hollingsworth lab directly from the originator (Dr. Iwamura) in the early 1990s. The S2-028 cells used in the studies described herein were approximately passage 40. Similarly, the T3M-4 cell line — used at approximate passage 50 — was a direct gift from the originator (Dr. Okabe) to Dr. Hollingsworth's mentor (R. Metzgar) at the Duke University Medical Center in the early 1980s. Both of these cell lines were authenticated by STR profiling in 2009 and 2014, respectively.

muAR9.6 Expression and Purification

The hybridoma was thawed in 100% FCS followed by a period of 2 weeks to transition from being cultured in serum-containing medium to serum free medium (12045076; Gibco) in T75 flasks. Once acclimatized to growing in serum free medium, the hybridoma cells were seeded in a Corning HYPER*Flask* (10024; Corning Life Sciences) and left to incubate for 4 weeks. After 4 weeks, the cell culture supernatant was gently decanted under a laminar flow hood into autoclaved bottles and centrifuged in an Avanti Beckman Coulter high speed centrifuge at a speed of 8000 rpm for 20 mins. The supernatant was carefully decanted under a laminar flow hood and diluted with 4× (v/v) in autoclaved phosphate buffered saline (PBS) containing no calcium and magnesium. Affinity chromatography was used to purify muAR9.6 IgG by passing the diluted muAR9.6 hybridoma supernatant at a flow rate of 0.5 ml/min over a HiTrap protein G HP column (29048581; Cytiva) hooked onto an AKTAprime plus FPLC instrument (11001313; Cytiva). Fractions corresponding to a single peak of high A_{280} absorbance were pooled together, quantified for IgG using a BioSpec-Nano spectrophotometer (Shimadzu), and verified for the presence and purity of IgG via SDS-PAGE.

Flow Cytometry

To evaluate the MUC16-binding ability of muAR9.6 and huAR9.6, flow cytometry experiments were performed with MUC16-positive OVCAR3 and MUC16-negative SKOV3 human ovarian cancer cells. 1×10^6 OVCAR3 or SKOV3 cells were treated under 3 conditions, two of which were controls. The first control was unstained cells. The second control was cells that were incubated with goat anti-mouse (for muAR9.6) or goat anti-human (for huAR9.6) Alexa Fluor 568-labeled secondary antibodies (A-11004 and A-21090 respectively; Thermo Fisher Scientific) for 30 minutes on ice. In the experimental group, the cells were incubated with 6.25 μg/mL unmodified muAR9.6 or huAR9.6 for 30 min on ice followed by rinses with ice cold PBS pelleting, resuspension and incubation with an Alexa Fluor 568-labeled secondary antibody for 30 minutes on ice. Following three more rinses with ice-cold PBS, pelleting, and resuspension, 1:1000 dilution of DAPI was added to the cell suspension, and the cells were analyzed on a BD LSR II (BD Biosciences, San Jose, CA) with 10,000 events gated per sample. Binding data was collected in triplicate, averaged, and plotted using FlowJo software.

Immunostaining and Fluorescence Microscopy

Upon splitting from 80% confluent T150 flasks, OVCAR3 cells were grown separately on sterile glass coverslips placed in 6-well tissue culture plates. Cells were seeded at a density of 250,000 cells in 3 mL growth medium per well. The cells were incubated for 48 h in a sterile incubator set to 37 °C and supplied with 5% CO_2. Thereafter, the cells were rinsed with PBS and fixed in 100% methanol for 30 min at -20 °C. A fluorescein isothiocyanate (FITC)-labeled version of the muAR9.6 antibody was generated using the Pierce FITC antibody labeling kit (53027; Thermo Fisher Scientific). The fixed cells were incubated in 5% non-fat dry milk (Carnation) in PBS and stained separately for 1 h with 50 µL of a 1:250 dilution of unmodified muAR9.6 (3.0 mg/mL) or FITC-labeled muAR9.6 (2 mg/mL) spotted on parafilm. 50 µL of a 1:500 dilution of Alexa-Fluor® 488-conjugated goat anti-mouse antibody (1 mg/mL) (A-11001; Thermo Fisher Scientific) suspended in PBS containing 5% non-fat dry milk was used as a secondary antibody. All antibody incubations were followed by three rinses using 0.1% PBST (0.1% Tween-20-supplemented phosphate-buffered saline) for 10 min each. Upon completion of the incubations and washes, the coverslips were mounted on microscopy slides (Fisherbrand) using Mowiol® mounting medium (475904; Calbiochem) supplemented with DAPI (50 µg/ml). Immunofluorescence was observed through a Zeiss Plan Apochromat 63× objective with a 1-1.25× tube lens on an axioscope fluorescence microscope.

Antibody Conjugation

3 mg of muAR9.6 and huAR9.6 antibodies (resuspended at concentrations > 2 mg/mL) were aliquoted, and the pH of the solution was adjusted to 8.7-9.0 using 1 M sodium carbonate (Na_2CO_3) followed by the drop-wise addition of 10 molar equivalents of isothiocynato-desferrioxamine (*p*-SCN-Phe-DFO) (B-705; Macrocyclics Inc.) dissolved to a concentration of 10 mg/mL in DMSO (41640; Sigma Aldrich). The volume of DMSO in the conjugation reaction was kept to less than 2% (v/v). The reaction mixture was incubated at 37 ºC for 1 h with constant shaking at 500 rpm in a thermomixer. Thereafter, the antibody-DFO conjugates were purified from excess unconjugated DFO using PD-10 desalting columns (17085101, Cytiva). The antibody-DFO conjugates collected in the 2 mL elution fraction were subsequently concentrated to 3-5 mg/mL using Ultra-2 Amicon 50 kDa molecular weight cutoff (MWCO) filtration spin columns (UFC205024, Millipore).

Radiolabeling with ^{89}Zr

High specific activity zirconium-89 (^{89}Zr-oxalate) was procured from 3D Imaging (Little Rock, AR, USA) or produced at Memorial Sloan Kettering Cancer Center on a TR19/9 cyclotron (Ebco Industries Inc.). Activity measurements were made using a CRC-15R Dose Calibrator (Capintec). ^{89}Zr[Zr]-oxalate was neutralized using 1 M sodium carbonate, and — depending on the scale of the study being conducted (the number of animals to be injected for PET imaging and biodistribution studies) — ^{89}Zr-radioimmunoconjugates were prepared by mixing 41–163 MBq (1.1 – 4.4 mCi) of pH-adjusted $^{89}Zr[Zr]^{4+}$ with 150–870 μg of each of DFO-conjugated AR9.6 suspended in Chelex-treated PBS (pH 7.2). The mixture was incubated with gentle stirring for 1 h at 25 °C in a reaction volume of 300–700 μL to achieve a radioactivity concentration between 2.5–9 μCi/μL. The reaction progress was assayed via radio instant thin layer chromatography (radio-ITLC) on an AR-2000 Bioscan using silica-impregnated paper (ITLC-SG, Varian) with an eluent of 50 mM EDTA (pH 5). After 1 h, the radiolabeling reaction was quenched by adding 1/10 (v/v) 10 mM EDTA. Finally, the radioimmunoconjugates were purified using a PD-10 desalting column for size-exclusion chromatography equilibrated with Chelex-treated PBS (dead volume 2.5 mL and elution volume 2 mL). The purity of the radioimmunoconjugate preparation was assayed by radio-ITLC.

Radioligand Binding Assay

The MUC16 binding activity of [^{89}Zr]Zr-DFO-muAR9.6 was validated via an *in vitro* radioligand binding assay using MUC16-positive OVCAR3 and MUC16-negative SKOV3 cells. To this end, a 0.15 μg/mL solution of [^{89}Zr]Zr-DFO-muAR9.6 was prepared in Chelex-treated PBS supplemented with 1% BSA. Twenty microliters of the radioimmunoconjugate solution were added to a microcentrifuge tube containing 50×10^6 cells in 1 mL of culture media. The resulting mixture was incubated for 1 h on ice with intermittent tapping to resuspend the cells. Thereafter, the cells were pelleted via centrifugation (600 × g for 3 min), and the supernatant was pipetted into a separate microcentrifuge tube. The cells were washed with 1 mL of ice-cold PBS, and centrifuged (600 × g for 3 min) before pipetting out the supernatant to another centrifuge tube. Two such washes were repeated. Finally, the cell pellet, the media supernatant, and the three wash fractions were measured for radioactivity on a gamma counter calibrated for ^{89}Zr. The percentage of cell

bound radioactivity of the radioimmunoconjugate was determined using the formula: Immunoreactive Fraction = [$Counts_{Cell\ Pellet}$]/[[$Counts_{Cell\ Pellet}$ + $Counts_{Media\ Supernatant}$ + $Counts_{Wash1}$ + $Counts_{Wash2}$ + $Counts_{Wash3}$]

Cell Internalization Assay

The MUC16 binding activity of [^{89}Zr]Zr-DFO-muAR9.6 and [^{89}Zr]Zr-DFO-huAR9.6 was validated via an *in vitro* cell binding and internalization assay using MUC16-positive OVCAR3 cells. To this end, 1 million OVCAR3 cells were plated per well in 6-well plates and incubated overnight in 2 mL of OVCAR3 cell growth media per well. Next day, huAR9.6 was radiolabeled with Zr-89 and a 10 ng/mL solution (15 mL) of [^{89}Zr]Zr-DFO-mu/huAR9.6 was prepared in cell growth media. Similarly, a 15 mL solution of 10 ng/mL of [^{89}Zr]Zr-DFO-mu/huAR9.6 was prepared having 10 μg/mL of unlabeled mu/huAR9.6. The latter solution was used to quantify binding and internalization of [^{89}Zr]Zr-DFO-mu/huAR9.6 in a 1000-fold excess of unlabeled huAR9.6.

Media from 6 well plates of OVCAR3 cells incubated overnight was aspirated, the cells were briefly rinsed with 1 mL of sterilized PBS followed by addition of 1 mL of the unblocked versus blocked [^{89}Zr]Zr-DFO-huAR9.6 solutions to each well in the 6 well plates. Each designated 6 well plate was incubated for the following periods of time – 1 h, 3 h, 6 h and 24 h in a sterile cell culture incubator set to 37°C and providing humidified air mixed with 5% CO_2. At the end of each designated period of incubation, the cell media was aspirated using a pipette and transferred to an 1.5 mL microcentrifuge tube labeled 'Sup'. Each well was briefly rinsed twice with 1 mL of sterile PBS and the supernatant was collected into a microcentrifuge tubes labeled 'Wash-1 and Wash-2' respectively. The amount of activity in the supernatant (Sup + Wash-1 + Wash-2) would be indicative of radioactivity bound to shed MUC16 and the fraction not bound to the OVCAR3 cells. Thereafter, 1 mL of freshly prepared 50 mM glycine-HCl/150 mM NaCl, pH 2.8 was added to each well and the cells were incubated for 5 minutes at room temperature. The supernatant from the low pH solution treatment of cells was aspirated using a micropipette and transferred to a 1.5 mL microcentrifuge tube labeled "Acid-1". The acid treatment was repeated, and the supernatant was collected and labeled "Acid-2". The amount of activity in the acid washes (Acid-1 + Acid-2) would be indicative of radioactivity bound to OVCAR3 cell membrane at the timepoint when this treatment was administered. Next, the cells were briefly rinsed with PBS followed by treatment

with a 1 mL solution of 1M NaOH via incubation for 10 minutes at room temperature. The supernatant thereof was aspirated using a micropipette and collected in a microcentrifuge tube labeled "Cell". The latter would indicate the amount of radioactivity internalized by the cells at the timepoint when this treatment was administered. At each of the indicated timepoints, a set of 6 well plates were maintained for the sole purpose of estimating the number of cells remaining at each timepoint. Finally, each of the microcentrifuge tubes containing fractions collected from the assay were measured for radioactivity on a gamma counter calibrated for ^{89}Zr. The percentage of radioactivity internalized at each timepoint was determined using the formula: Internalized Fraction = $[Counts_{Cell}]/[[Counts_{Sup} + Counts_{Wash\text{-}1} + Counts_{Wash\text{-}2} + Counts_{Acid\text{-}1} + Counts_{Acid\text{-}2} + Counts_{Cell}]$

Size Exclusion-High Performance Liquid Chromatography (SE-HPLC)

SE-HPLC was performed to evaluate the monomeric content of the unmodified muAR9.6 and huAR9.6 prior to and after conjugation to DFO. A Phenomenex Yarra 3 μm SEC-3000 (300 × 7.8 mm) column was used on an HPLC system (Shimadzu Scientific Instruments, Inc.). The mobile phase used was an aqueous solution containing 1× phosphate-buffered saline pH 7.2. The flow rate was set to 1 mL/min and the run time was 20 minutes. Peaks on the 280 nm chromatogram were integrated using LabSolutions Postrun software (Shimadzu Scientific Instruments, Inc.).

Radio Size Exclusion-High Performance Liquid Chromatography (Radio-SE-HPLC)

SE-HPLC was performed to evaluate the stability of [^{89}Zr]Zr-DFO-muAR9.6 and [^{89}Zr]Zr-DFO-huAR9.6. To this end, 33 μL of each of the radioimmunoconjugates (50 μg, 500 μCi) were added to 450 μL of human serum (H4522; Sigma Aldrich). And as a control, free [^{89}Zr]Zr^{4+} (28 μL, 800 μCi) was added to 750 μL of human serum. The samples were agitated constantly on a thermomixer set to 37 °C. Subsequently, samples were taken from each microcentrifuge tube and analyzed via radio-SE-HPLC. A Superdex 200 Increase 10/300 GL column (Cytiva, Marlborough, MA, USA) was used on an HPLC system (Shimadzu Scientific Instruments, Inc) with a mobile phase of 1× phosphate-buffered saline (pH 7.2), a flow rate of 0.75 mL/min, and a run time of 35 minutes. The absorbance was monitored at 280 nm, and peaks were integrated using LabSolutions Postrun software (Shimadzu Scientific Instruments, Inc).

Western Blotting

OVCAR3, SKOV3, T3M4, MiaPaCa-2, Capan-2, S2028, and BxPC3 cell lysates were prepared using radioimmunoprecipitation assay [RIPA] lysis and extraction buffer (Thermo Fisher Scientific; 89900). The cell lysates were quantified for protein content using a BCA assay (Thermo Fisher Scientific; 23235). 25 µg of protein from each cell lysate was loaded in a separate well and resolved on a NuPAGE 3–8% Tris-Acetate SDS-Page gel (ThermoFisher Scientific; EA0375BOX) and transferred to polyvinylidene fluoride (PVDF) membrane (Thermo Fisher Scientific; 88520) using iBLOT 2 dry blotting system (Thermo Fisher Scientific; IB21001). The membranes were probed with the following primary and secondary antibodies: anti-MUC16 (muAR9.6), HRP-linked anti-β-actin, and HRP-linked anti-mouse IgG (Cell Signaling Technology, Danvers, MA, USA). Novex Sharp Pre-stained Protein Standard (Thermo Fisher Scientific; LC5800) was used to identify the molecular weight of the protein bands on the gel. ImageJ was used to perform densitometric analysis on the high and low molecular weight bands of MUC16 epitopes that muAR9.6 bound to and the actin loading control band.

Serum Stability

The *in vitro* stability of the purified radioimmunoconjugates ([^{89}Zr]Zr-DFO-muAR9.6 and [^{89}Zr]Zr-DFO-huAR9.6) were tested for demetallation of [^{89}Zr]Zr^{4+} by incubation in human AB-type serum. 100 µL of each of the radioimmunoconjugates (5 µg, 45 µCi of [^{89}Zr]Zr-DFO-muAR9.6 having a specific activity of 9 µCi/µg versus 31 µg, 155 µCi of [^{89}Zr]Zr-DFO-huAR9.6 having a specific activity of 5 µCi/µg) was incubated with 900 µL of human serum (H4522; Sigma Aldrich) and agitated constantly on a thermomixer set at 37 °C. Samples were taken from each microcentrifuge tube and analyzed via radio-iTLC by spotting them on the baseline of silica-gel impregnated glass-microfiber paper strips (iTLC-SG, Varian, Lake Forest, CA) and dipping the bottom of the iTLC strips in a vertical TLC chamber containing EDTA mobile phase, 50 mM, pH 5. Once the solvent had traveled up the far end of the strip, the iTLC strips were analyzed by placing them on an AR-2000 iTLC scanner (Bioscan Inc., Washington, DC) and area under the curves at the baseline vs. solvent front were recorded. This process was repeated for each sample (triplicates) every day out to day 6 post-incubation in human AB type serum. The serum stability of the radioimmunoconjugates was measured as the percentage of [^{89}Zr]Zr^{4+} retained at the origin of the radio-ITLC strip and reported as % intact.

PET Imaging

Animals were anesthetized by inhalation of 2% isoflurane (Baxter Healthcare) and medical air gas mixture and placed within the scanner with anesthesia maintained using 2% isoflurane and medical air gas mixture. PET-CT data for each mouse were recorded via static scans every 24 hours post-injection (p.i.). An energy window of 350-700 keV and a coincidence timing window of 6 ns were used. Data were sorted into 2D histograms by Fourier rebinning, and transverse images were reconstructed by filtered back-projection (FBP) into a 128 × 128 × 63 (0.72 × 0.72 × 1.3 mm^3) matrix. The counting rates in the reconstructed images were converted to activity concentrations (percentage injected dose per gram of tissue, %ID/g) by use of a system calibration factor derived from the imaging of a mouse-sized water-equivalent phantom containing ^{89}Zr.

Ex vivo Biodistribution

Animals were euthanized by CO_2 asphyxiation at predetermined experimental timepoints between days 1 and 6 after the administration of ^{89}Zr-labeled AR9.6 radioimmunoconjugates. Following euthanasia, several organs of interest were collected, weighed, and assayed for radioactivity on a gamma counter calibrated for ^{89}Zr. Counts were converted into activity using a calibration curve generated from known standards. Count data were background and decay corrected to the time of injection, and the percent injected dose per gram (%ID/g) for each tissue sample was calculated by normalization to the total activity injected.

Histopathology

In addition to analyzing tissue sections for morphology, histopathologic analysis of tissues was performed using immunohistochemistry (IHC). IHC staining of cryosections of ovarian patient tumors was done using muAR9.6 as the primary antibody and a biotinylated goat anti-mouse (H+L) secondary antibody (BP-9200; Vector labs). IHC staining of OVCAR3 tumors and lymph node sections was performed using huAR9.6 as the primary antibody and a biotinylated goat anti-human (H+L) secondary antibody (BA-3000; Vector labs). Pancytokeratin staining of sections from OVCAR3 tumors and lymph nodes was performed using a rabbit polyclonal antibody (Z0622; Dako) as the primary antibody and a biotinylated anti-rabbit IgG (H+L) (PK-6100; Vector labs) as the secondary antibody.

Slides with frozen sections of ovarian patient tumor samples were air-dried for 30 min and fixed in 10% buffered formalin for 10 min and rinsed with 3 changes of PBS. Slides with formalin-fixed paraffin-embedded (FFPE) sections were heated at 58-60°C for 30 min before deparaffinization in xylene and hydration in graded alcohols and water. Endogenous peroxidase activity was blocked by incubation in 1% hydrogen peroxide in PBS for 15 min and 3 rinses with PBS alone. This step was followed by unmasking of the antigenic epitopes by steaming in 10 mM citrate buffer for 15 min. Before further processing, the slides were cooled for 20 min, washed in distilled water for 5 min and transferred to PBS. This step was followed by incubation in 10% normal goat serum (55984; MP Biomedicals) or donkey serum (017-000-121; Jackson Immuno Research) diluted in 2% BSA-PBS diluent for 30 min in a humid chamber. The only difference in the AR9.6 versus pan-cytokeratin IHC staining was that the AR9.6 monoclonal antibody was used at a 1:100 dilution whereas the pan-cytokeratin polyclonal antibody was applied at 1:500 dilution. After application of the primary antibody, the slides were incubated overnight at 4 °C in a humid chamber. Next day, the slides were rinsed with 3 changes of PBS followed by application of the secondary antibodies diluted 1:500 in 1% BSA-PBS for 1 hour at room temperature. Thereafter, 3 changes of PBS were done, and the slides were treated with a 1:25 dilution of the avidin-biotin complex (PK-6100; Vector labs) for 30 min at room temperature. The slides were then incubated in 3,3-diaminobenzidine substrate (AAH5400014; Fisher Scientific) until the desired color intensity was achieved. Counterstaining was performed with hematoxylin. Further rinsing of the slides was done under running tap water followed by dehydration with 3 changes each of 95% alcohol, 100% alcohol, and xylene before applying the coverslip.

Humanization of muAR9.6

Candidate human antibody genes for CDR grafting were selected by alignment of the murine AR9.6 VH and Vκ sequences against the IMGT human antibody germline database (1). Sequences with the highest degree of homology and similarity in terms of CDR canonical structures were selected for grafting. The human germline genes IGHV3-48 and IGHJ4*01 were selected for the VH, and germline genes IGKV3D-11 and IGKJ4*01 were used for Vκ CDR grafting. Homology models of grafted humanized Fv were constructed and based on structural analysis of the

homology models, murine framework residues within the Vernier zone deemed important for maintaining CDR structure were selected for back mutation. In the heavy chain, a single residue was selected for while within the light-chain, three residues were chosen for back-mutation. The sequence for the final humanized AR9.6 Fv was codon-optimized for expression in CHO cells, and the genes coding for an intact IgG1 heavy and light chains were produced by gene synthesis (Life Art, ThermoFisher Scientific) and cloned into pcDNA3.4 (ThermoFisher Scientific).

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