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CD46-Targeted Theranostics for PET and ²²⁵Ac-Radiopharmaceutical Therapy of Multiple Myeloma



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ABSTRACT

Purpose: Multiple myeloma is a plasma cell malignancy with an unmet clinical need for improved imaging methods and therapeutics. Recently, we identified CD46 as an overexpressed therapeutic target in multiple myeloma and developed the antibody YS5, which targets a cancer-specific epitope on this protein. We further developed the CD46-targeting PET probe [⁸⁹Zr]Zr-DFO-YS5 for imaging and [²²⁵Ac]Ac-DOTA-YS5 for radiopharmaceutical therapy of prostate cancer. These prior studies suggested the feasibility of the CD46 antigen as a theranostic target in multiple myeloma. Herein, we validate [⁸⁹Zr]Zr-DFO-YS5 for immunoPET imaging and [²²⁵Ac]Ac-DOTA-YS5 for radiopharmaceutical therapy of multiple myeloma in murine models.

Experimental Design: *In vitro* saturation binding was performed using the CD46 expressing MM.1S multiple myeloma cell line. ImmunoPET imaging using [⁸⁹Zr]Zr-DFO-YS5 was performed in immunodeficient (NSG) mice bearing subcutaneous and systemic multiple myeloma xenografts. For radioligand therapy, [²²⁵Ac]Ac-DOTA-YS5 was prepared, and both dose escalation and fractionated dose treatment studies were performed in mice bearing MM1.S-Luc systemic xenografts. Tumor burden was analyzed using BLI, and body weight and

overall survival were recorded to assess antitumor effect and toxicity.

Results: [⁸⁹Zr]Zr-DFO-YS5 demonstrated high affinity for CD46 expressing MM.1S multiple myeloma cells ($K_d = 16.3$ nmol/L). *In vitro* assays in multiple myeloma cell lines demonstrated high binding, and bioinformatics analysis of human multiple myeloma samples revealed high CD46 expression. [⁸⁹Zr]Zr-DFO-YS5 PET/CT specifically detected multiple myeloma lesions in a variety of models, with low uptake in controls, including CD46 knockout (KO) mice or multiple myeloma mice using a nontargeted antibody. In the MM.1S systemic model, localization of uptake on PET imaging correlated well with the luciferase expression from tumor cells. A treatment study using [²²⁵Ac]Ac-DOTA-YS5 in the MM.1S systemic model demonstrated a clear tumor volume and survival benefit in the treated groups.

Conclusions: Our study showed that the CD46-targeted probe [⁸⁹Zr]Zr-DFO-YS5 can successfully image CD46-expressing multiple myeloma xenografts in murine models, and [²²⁵Ac]Ac-DOTA-YS5 can effectively inhibit the growth of multiple myeloma. These results demonstrate that CD46 is a promising theranostic target for multiple myeloma, with the potential for clinical translation.

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Introduction

Multiple myeloma is the second most common hematologic malignancy among adults, with >33,000 new cases diagnosed per year in the United States (1). Multiple myeloma is a highly heterogeneous disease, and distinct subtypes of this disease can result in different outcomes and clinical-pathological features. Despite tremendous progress in multiple myeloma treatment in recent decades, there is no known cure (2). Patients typically endure cycles of initial response but subsequent resistance and relapse after treatment with different therapeutic agents (3). In particular, patients with extramedullary disease (i.e., myeloma tumor established outside of the typical location within the bone marrow) are considered high risk with no established treatment strategy (4). This indication in particular has an urgent need for novel therapeutic strategies. The current imaging approach for multiple myeloma includes radiographic skeletal surveys, whole-body low-dose CT, whole-body MRI, and 2-deoxy-2-[¹⁸F]-fluoro-deoxyglucose ([¹⁸F]-FDG) PET (5, 6). These modalities have complementary roles, with MRI being highly sensitive for bone marrow disease, and [¹⁸F]-FDG PET being most useful for detecting extramedullary disease as well as for assessing response to treatment (7, 8). However, [¹⁸F]-FDG exhibits limitations including high background uptake in tissues like brain and bone marrow, false positives due to

Translational Relevance

There is an unmet clinical need for improved imaging methods for detecting multiple myeloma and improved treatments. Recently, we identified CD46 as an overexpressed therapeutic target in multiple myeloma and have successfully developed the antibody YS5, which specifically targets a cancer-specific epitope on this protein. Herein, we report the preclinical evaluation of CD46-directed theranostics in multiple myeloma. [⁸⁹Zr]Zr-DFO-YS5 was evaluated for immunoPET imaging of multiple myeloma in different murine models, which demonstrate specific, target-mediated imaging with a high tumor uptake. Further, we have performed radiopharmaceutical therapy with [²²⁵Ac]Ac-DOTA-YS5 in a systemic model of multiple myeloma, with a marked reduction in tumor burden and improvement in overall survival. These results highlight the efficacy of CD46 targeted theranostics in multiple myeloma and strongly support future clinical translation.

inflammation or osteoarthritis, and false negatives due to low expression of hexokinase in some multiple myeloma lesions (9). Therefore, there is an unmet clinical need for improved imaging methods to detect multiple myeloma.

With the recent FDA approval of [¹⁷⁷Lu]Lu-DOTATATE (Lutathera) and [¹⁷⁷Lu]Lu-PSMA-617 (Pluvicto), theranostics promise to change the standard of care in multiple cancer types (10–12). The basic therapeutic strategy requires targets that are overexpressed in cancer cells but not in normal tissues. A key advantage of this approach is the paired utilization of molecular imaging (typically with PET) together with radiopharmaceutical therapy using analogous molecules. Studies using CXCR4 (13–15), CD38 (16–18), CD56 (19, 20), CD158 (21), or CD20 (22) as theranostic targets have shown success in multiple myeloma imaging and therapy in preclinical studies and in clinical trials. Of these theranostic targets, CXCR4 has attracted significant attention in both solid tumors and multiple myeloma, with great promise in clinical trials and preclinical models (23–25). In addition, clinical trials for radioimmunotherapy (RIT) with [¹¹¹In]In/[²²⁵Ac]Ac-DOTA-Daratumumab targeting CD38 are ongoing (NCT053631111). However, despite these advances, none of these agents have yet risen to the standard of care, and there remains an unmet need for improved theranostic strategies for the imaging and treatment of multiple myeloma.

CD46 is a recently identified therapeutic target for multiple myeloma (26). It is located on chromosome 1q, which is commonly amplified in relapsed patients with multiple myeloma. In 2016, we reported that CD46 expression was markedly higher in multiple myeloma cells with 1q copy gain, a high-risk subtype of multiple myeloma, and identified a human monoclonal antibody (23AG2, aka YS5; ref. 26) binding to a tumor-selective CD46 epitope (27). We then developed an antibody–drug conjugate by conjugating auristatin derivatives [monomethyl auristatin F (MMAF) and monomethyl auristatin E (MMAE)] to YS5 and found strong *in vitro* and *in vivo* antitumor activity across multiple myeloma preclinical models (27). This MMAE-conjugated ADC (FOR46) is now in a multicenter phase I clinical trial for multiple myeloma (NCT03650491), as well as prostate cancer, with promising initial results reported (28). In parallel, we have successfully developed a CD46-targeting PET probe [⁸⁹Zr]Zr-DFO-YS5 for prostate cancer imaging with high sensitivity and validation in numerous preclinical models (29). A clinical trial of this imaging agent is now enrolling men with prostate cancer (NCT05245006).

Similarly, we found promising therapeutic results in prostate cancer models, using the targeted alpha particle nanogenerator [²²⁵Ac]Ac-DOTA-YS5 (30). Taken together, these results demonstrate great promise for the use of CD46-directed theranostics in multiple myeloma using the YS5 antibody as a platform.

Herein, we report the development and initial evaluation of CD46-directed theranostics in multiple myeloma. [⁸⁹Zr]Zr-DFO-YS5 was evaluated for immunoPET imaging of multiple myeloma in murine models, which demonstrates a key finding of specific, target-mediated imaging with a high tumoral uptake. Moreover, radiopharmaceutical therapy with [²²⁵Ac]Ac-DOTA-YS5 was tested in a systemic model of multiple myeloma, with a marked reduction in tumor burden and improvement in overall survival. Taken together, these results demonstrate the efficacy of CD46 theranostics in multiple myeloma and strongly support future clinical translation.

Materials and Methods

General materials

The recombinant fully human CD46-targeted antibody, YS5, was generated from mammalian cell expression and purified as described previously (26). ⁸⁹Zr oxalate was purchased from 3D Imaging (Little Rock) and the Cyclotron Laboratory at the University of Wisconsin. ²²⁵Ac was purchased from the Department of Energy Isotope Production Program and produced at Oak Ridge National Laboratory via the ²²⁹Th generator route. p-Isothiocyanate-benzyl-DFO (Catalog No. B-705) and p-Isothiocyanate-benzyl-DOTA (Catalog No. B-205) were purchased from Macrocytics, Inc. Native human IgG (Catalog No. ab91102) was purchased from Abcam. iTLC-SG (Catalog No. SG10001) was purchased from Agilent Technologies.

Antibody conjugation and radiolabeling

DFO-YS5, DFO-IgG, and [⁸⁹Zr]Zr-DFO-YS5 and [⁸⁹Zr]Zr-DFO-IgG were synthesized as reported previously (29). Conjugation of antibody YS5 as well as IgG with DOTA and further radiolabeling with ²²⁵AcNO₃ was carried out by following previously reported procedures (30). The dose calibrator details to measure the activity of the radioisotopes (⁸⁹Zr and ²²⁵Ac) have been included in the Supplementary Materials and Methods.

Cell culture

MM.1S, RPMI8226, and AMO1 cell lines were obtained from the ATCC. The accession numbers for the MM.1S, RPMI8226, and AMO1 cell lines are CRL-2974, CCL-155, and ACC 538, respectively. The ANBL6 cells were obtained from Dr. Diana Jelinek (Mayo Clinic; RRID:CVCL_5425). MM.1S cells, RPMI8226 cells, AMO1, and ANBL6 cells were maintained in RPMI1640 medium supplemented with 20% FBS, 100 U penicillin, and 100 µg/mL streptomycin in a humidified incubator at 37°C and 5% CO₂. ANBL6 cells were grown in RPMI1640 medium supplemented with 20% FBS, 100 U penicillin, 100 µg/mL streptomycin, and 2 ng/mL IL6 in a humidified incubator at 37°C and 5% CO₂ (31). Cell lines used in our studies were negative for mycoplasma contamination when tested with bioluminescence-based MycoAlert Mycoplasma Detection Kit in our UCSF cell culture lab.

Generation of luc+ cells

All cell lines MM.1S, RPMI8226, ANBL6, AMO1, and MM.1S CD46 (–/–) were all modified to stably express luciferase according to a previously established procedure and transduced with a lentiviral vector expressing mCherry fluorescent tag (32). Tumor burden was assessed through noninvasive bioluminescence.

Cas9 knockouts of MM.1S cells

Cas9 protein and sgRNA or scrambled sgRNA were mixed in a 1:1 molar ratio (Synthego Corporation) and incubated at 37°C for 10 to 15 minutes. MM.1S cells (1 million) were spun down and washed with PBS, resuspended in 20 μ L of SF nucleofection/solution plus cas9/sgRNA mixture (SF Cell Line 4D-Nucleofector X Kit S; Lonza), and nucleofected using the DS-137 nucleofection program in Lonza 4D-Nucleofector. Eighty microliters of warm RPMI1640 media (Gibco) supplemented with 20% of FBS, GlutaMAX, and 100 U penicillin, 100 μ g/mL streptomycin was plated into each well and incubated at 37°C for 15 minutes, then transferred to a 6-well to recover for 48 hours. Then, based on the surface expression by flow cytometry, negative clones were sorted using FACSARIA II flow cytometer (BD Biosciences). sgRNA sequences were obtained from the Brunello Library (33) as follows: CD46: AUUGUGGUGACA-AUUCAGUG; negative control, scrambled sgRNA#1 and #2 mod-sgRNA specified by Synthego Corporation.

Computational epigenetic analysis

Methods for computational epigenetic analysis to identify possible transcriptional regulators of CD46 have been included in the Supplementary Materials and Methods.

In vitro K_d measurement

The K_d value of [⁸⁹Zr]Zr-DFO-YS5 against CD46-expressing cell line MM.1S was determined by a saturation binding assay. An aliquot of 5×10^5 MM.1S cells was added to each of 18 0.5 mL centrifuge vials. The cells were centrifuged, and the supernatant was removed. The pellet was washed with PBS twice. Various concentrations (200 μ L/vial, 0.0005–50 nmol/L, triplicate) of [⁸⁹Zr]Zr-DFO-YS5 in PBS containing 1% nonfat milk were added to cells. The cells were incubated with [⁸⁹Zr]Zr-DFO-YS5 containing buffer for 1 hour at 37°C. After that, the cells were centrifuged, and the supernatant was removed. The pellet was washed with PBS twice. The radioactivity bound to the cell pellet was counted using a Hidex gamma counter. K_d value and B_{max} were calculated by nonlinear regression, one site-specific binding using GraphPad Prism Software (GraphPad Software).

Flow cytometry

Cells were resuspended in FACS buffer (2% FBS in D-PBS) and stained with CD46 (BioLegend, clone MEM-258) and CD138 (BioLegend, clone DL-101) antibodies for 30 minutes at 4°C, washed, and then resuspended in FACS buffer. For experiments where live cell populations were analyzed by flow cytometry with anti CD46-YS5 antibody, cells were resuspended in 100 μ L of FACS buffer, labeled with 0.5, 1, or 2 μ g of YS5 antibody, incubated for 30 minutes at 4°C and then stained with AffiniPure F(ab')₂ Fragment Goat Anti-Human IgG, Fc Fragment Specific (Jackson ImmunoResearch Laboratories Inc.) at 1:50 concentration as recommended per manufacturer, washed and resuspended in FACS buffer. Samples were analyzed using either a Cytoflex Flow Cytometer (Beckman Coulter) or FACSARIA II flow cytometer (BD Biosciences). Data analysis was done using FlowJo software, v10.8.1, RRID:SCR_008520. Antibodies used were FITC anti-human CD46 Antibody (BioLegend 315304, RRID:AB_2291290; 0.013 nmol/L), APC anti-human CD138 (Syndecan-1) Antibody (BioLegend 352308, RRID:AB_10896946; 0.013 nmol/L), FITC Mouse IgG1, κ Isotype Ctrl Antibody (BioLegend 400108, RRID:AB_400108; 33 nmol/L), APC Mouse IgG1, κ Isotype Ctrl (BioLegend 981806; 0.013 nmol/L), and fluorescein (FITC)-conjugated AffiniPure F(ab')₂ Fragment Goat Anti-Human IgG, Fc Fragment Specific (minimal

cross-reaction to Bovine, Horse, and Mouse Serum Proteins; Jackson ImmunoResearch Laboratories Inc., 109–096–098; 100 nmol/L).

Cell binding assay

Cell numbers of MM.1S, RPMI8226, ANBL6, AMO1, or CD46-KO MM.1S were counted. Thirty million cells of each cell line were aliquoted to a 1.7 mL centrifuge vial (in triplicate). The cells were centrifuged at 1,000 revolutions per minute, and the supernatant was removed. The pellet was washed with PBS twice. Then 0.05 μ Ci [⁸⁹Zr]Zr-DFO-YS5 in 200 μ L PBS (final antibody concentration of 3.125 nmol/L) containing 1% nonfat milk was added to each vial. The cells were incubated in this buffer for 1 hour at 37°C. The cells were centrifuged, and the supernatant was removed. The pellet was washed with PBS twice. The radioactivity of the cell pellet and 0.05 μ Ci [⁸⁹Zr]Zr-DFO-YS5 was counted using a Hidex Gamma Counter using a 480 to 558 keV counting window. Cell-associated activity percentage was calculated by cell pellet activity/0.05 μ Ci [⁸⁹Zr]Zr-DFO-YS5 activity.

Animal studies-xenograft models

All animal studies were conducted according to Institutional Animal Care and Use Committee–approved protocols at the University of California (AN194778). To generate the subcutaneous multiple myeloma xenograft models, a mixture of male and female NOD SCID gamma (NSG, stock 005557, The Jackson Laboratory) mice ages 4 to 8 weeks old were inoculated subcutaneously with 10 million MM1.S-Luc, RPMI8226-Luc, or ANBL6-Luc cells in a 1:1 mixture (v/v) of cells in PBS and Matrigel (Thermo Fischer Scientific, 08774391). To generate systemic multiple myeloma xenograft models (also called disseminated, or orthometastatic), 5×10^5 MM1.S-Luc or RPMI8226-Luc cells in PBS were injected intravenously into a cohort of male and female NSG mice ages 4 to 8 weeks old. Bioluminescence imaging (BLI) was used to monitor graft status (typically injected tumor cells established themselves in the bone marrow of the femur close to joints in 10 days). Mice were subjected to PET imaging followed by biodistribution analysis when the subcutaneous tumor reached a size of 200 to 500 mm³ and in the systemic xenograft model when mice showed bioluminescence signal within a reference range of 1×10^6 to 6.06×10^6 p/sec/cm²/sr.

In vivo [⁸⁹Zr]Zr-DFO-YS5 and [¹⁸F]-FDG PET and [⁸⁹Zr]Zr-DFO-IgG imaging studies

Approximately 2 to 4 weeks after tumor implantation, animals with subcutaneous tumors reaching 200 to 500 mm³ or systemic multiple myeloma xenograft showing bioluminescence signal within a reference range 1×10^6 to 6.06×10^6 p/sec/cm²/sr were anesthetized by isoflurane inhalation. For [¹⁸F]-FDG PET/CT imaging, 7.5 to 10 MBq [200–220 μ Ci] of [¹⁸F]-FDG was administered ($n = 4$) and imaging methods were identical to those reported previously (34). For [⁸⁹Zr]Zr-DFO-YS5 PET imaging, 3.70 to 5.55 MBq [100–150 μ Ci, 10 μ g/mouse ($n = 4$)] of [⁸⁹Zr]Zr-DFO-YS5 and 0.5 mg nonspecific binding IgG, essential to block the Fc receptor in NSG mice in saline, was (35) administered through the tail vein. The animals were imaged at 6 days post-injection for the subcutaneous model or 4 days post-injection for the systemic model with a 20-minute acquisition time by using microPET/CT (Inveon; Siemens Medical Solutions). PET imaging data were acquired in list mode and reconstructed using an iterative 2D OSEM reconstruction algorithm (for Inveon data) provided by the manufacturer. The resulting image data were then normalized to the administered activity to parameterize images in terms of %IA/mL. Imaging data were viewed and processed using open-source Amide

software. CT images were acquired following PET for 10 minutes, and the CT data were used for attenuation correction for PET reconstruction and anatomic reference.

To validate the specificity of the tracer for multiple myeloma tumor targeting, three control studies were performed. PET imaging was performed 6 days after co-injection of [⁸⁹Zr]Zr-DFO-YS5 [5.55–6.29 MBq (150–170 μCi) (*n* = 5)] and 0.5 mg IgG with a group of mice bearing subcutaneous CD46 knockout (KO) MM.1S tumors. In a second blocking control study, a group of mice with MM.1S was co-injected with 0.5 mg unlabeled YS5, 0.5 mg IgG, and [⁸⁹Zr]Zr-DFO-YS5 [5.92–7.03 MBq (160–190 μCi)], followed by PET imaging 6 days after injection. In a nontargeting antibody control study, a control mAb IgG (nonbinding control) was also radiolabeled with ⁸⁹Zr ([⁸⁹Zr]Zr-DFO-IgG) in the same process and the PET imaging of mice bearing MM.1S xenograft was recorded 6 days after injection followed by a biodistribution study. PET imaging of healthy nude and NSG mice was recorded with [⁸⁹Zr]Zr-DFO-IgG and biodistribution was also performed at 4 days after injection.

BLI

In vivo BLI was performed to monitor the tumor progression in mice injected with luciferase-expressing myeloma cells. D-Luciferin (LUCK-1G-Gold Biotechnology; 150 mg luciferin/kg body weight) dissolved in 100 μL PBS was intraperitoneally injected into mice, and they were allowed to move freely. After 9 minutes, mice were anesthetized and imaged using the IVIS 50 instrument. Images were acquired after 60 seconds of exposure time, and the intensity of the luciferase signal was quantified as radiance (photons/sec/cm²/steradian) using an oval region of interest in Living Image 4.0 software.

For *ex vivo* bioluminescence studies, mice were injected intraperitoneally with D-Luciferin. After 5 minutes, mice were sacrificed, and organs were removed and plated in a petri dish. BLIs of the organs were acquired after 60 seconds of exposure time, and the intensity of the signal was calculated as described above.

Biodistribution studies

The tumor-bearing mice were sacrificed after PET/CT imaging. Blood was collected by cardiac puncture. For the subcutaneous model, major organs (bone, heart, kidney, large intestine, liver, lung, muscle, pancreas, small intestine, spleen, stomach, and subcutaneous tumor) were harvested. For the systemic model, bone marrow was extracted with some modifications in the previously established procedure (36). Briefly, a hole was created at the bottom of a 0.5 mL centrifuge tube using a needle. The femur was isolated from mice and was placed knee-end down in this 0.5 mL centrifuge tube. The 0.5 mL centrifuge tube was nested in a 1.7 mL centrifuge tube. Centrifuge the nested tubes at 10,000 rpm for 3 minutes. The bone marrow was collected in the 1.7 mL centrifuge tube. The organs collected were then weighed and counted in Hidex Gamma Counter in the 480 to 558 keV window. The percent injected activity per gram of tissue (%IA/g) was calculated by comparing with standards of known radioactivity.

Target binding fraction assay using magnetic beads

The target binding fraction assay for [²²⁵Ac]Ac-DOTA-YS5 was performed according to the prior published protocol (37). The bead's activity, supernatant, and standard (10 ng of [²²⁵Ac]Ac-DOTA-YS5) were measured on a Hidex gamma counter (energy window 25–2,000 keV). The binding fraction percentage was determined by calculating the bead's activity/standard.

Radioligand therapy for subcutaneous MM.1S tumor model

The detailed methodology for the radioligand therapy for the subcutaneous MM.1S tumor model has been included in the Supplementary Materials and Methods.

Radioligand therapy in the systemic MM.1S tumor model

Mice were administered 0.5 million MM.1S-Luc cells via the tail vein, and 7 days after injection, treatment was initiated. Mice were randomized to 6 treatment arms of *n* = 8 based on the average bioluminescence signal with a radiance value in between the range 1.40×10^4 p/sec/cm²/sr to 7.00×10^6 p/sec/cm²/sr. One mouse was kept separated from each group and used for the CD38 IHC. Treatment arms included a saline control, [²²⁵Ac]Ac-DOTA-YS5 at dose levels of 0.0625 μCi or 0.125 μCi, or [²²⁵Ac]Ac-DOTA-IgG at a dose level of 0.125 μCi. Additional fractionated dose arms of the study used administered activities of three fractions of 0.0625 μCi each and 0.125 μCi each of [²²⁵Ac]Ac-DOTA-YS5 at days 0, 28, and 56, respectively. Fc blocking with 0.5 mg of cold native IgG was also performed in all the [²²⁵Ac]Ac-DOTA-YS5 groups. Mice were monitored weekly via BLI as above. Body weight, activity, and overall mouse condition were monitored and analyzed at every third day. If body weight loss was ≥20% or mice demonstrated deteriorating conditions, including impaired motility or low body condition score (BSC < 2), they were euthanized. At day 120, the study was terminated with four mice alive. Overall survival was determined by Kaplan–Meier survival analysis. For the four surviving mice, animals were imaged with [⁸⁹Zr]Zr-DFO-YS5 PET/CT (as above), and tissues were harvested for toxicity analysis.

Necropsy and hematoxylin and eosin staining

The detailed methods for necropsy as well as for histology staining have been described in the Supplementary Materials and Methods.

IHC staining of CD138

The detailed methodology for IHC staining of CD138 has been added in the Supplementary Materials and Methods.

Statistical analysis

Data were analyzed using PRISM software (GraphPad; RRID: SCR_002798). The continuous variables were described by mean with SD and compared between two groups by using the unpaired and two-way ANOVA model. The survival data were estimated and described by the Kaplan–Meier method and were compared across groups with a log-rank sum test. Statistical significance was declared based on *P* < 0.05.

Data availability statement

The data presented in this study and in the Supplementary Information are available from the corresponding author upon request.

Results

CD46 is highly expressed in multiple myeloma, and can be detected using [⁸⁹Zr]DFO-YS5 in multiple myeloma cell lines *in vitro*

The binding affinity of [⁸⁹Zr]Zr-DFO-YS5 on the CD46-expressing multiple myeloma MM.1S cell line was verified through a saturation binding assay. Saturation binding analysis with [⁸⁹Zr]Zr-DFO-YS5 revealed a dissociation constant value, *K*_d of 16.5 ± 2.6 nmol/L, and a receptor density of 1.49×10^5 per cell in the MM.1S line (Fig. 1A). Further cell binding assays were performed to evaluate CD46 expression levels on different multiple myeloma cell lines. We found ANBL6

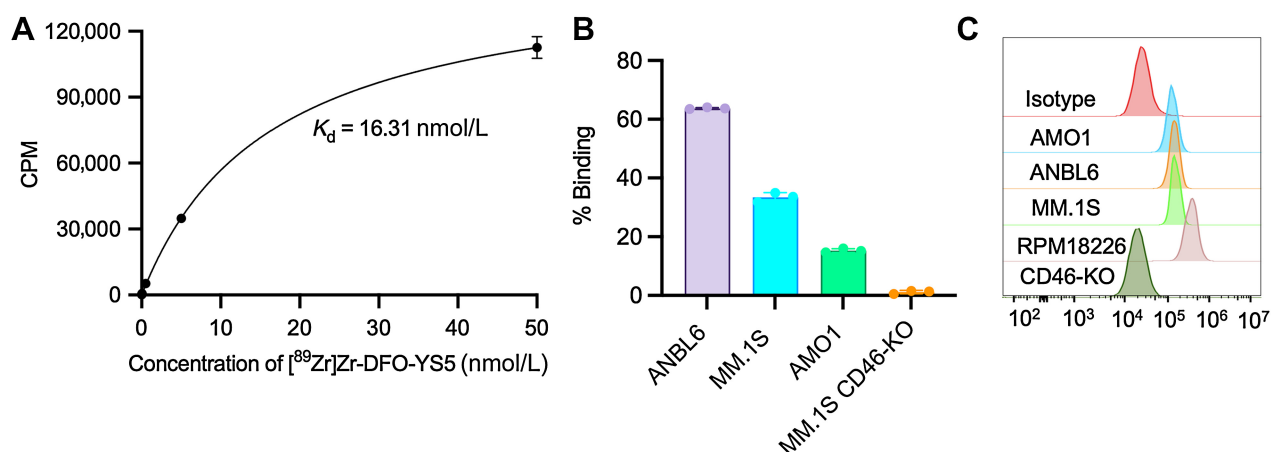


Figure 1.

$[^{89}\text{Zr}]\text{Zr-DFO-YS5}$ can detect CD46 expression in various multiple myeloma cell lines. **A**, K_d measurement of $[^{89}\text{Zr}]\text{Zr-DFO-YS5}$ on the MM.1S cell line ($n = 3$), determined by a saturation binding assay ($K_d = 16.31 \pm 2.6 \text{ nmol/L}$) with a receptor density of 1.49×10^5 per cell. **B**, Cell binding assay to measure the percent cell-associated activity of $[^{89}\text{Zr}]\text{Zr-DFO-YS5}$ using different multiple myeloma cell lines ($n = 3$). **C**, Flow cytometry analysis of CD46 cell surface expression in various multiple myeloma cell lines ($n = 3$).

to have the highest cell binding percentage of $[^{89}\text{Zr}]\text{Zr-DFO-YS5}$, followed by MM.1S and AMO1. In contrast, the CD46 negative control cell line CD46-KO MM.1S did not demonstrate significant binding of the radiopharmaceutical (Fig. 1B). Similar results were found when the assay was performed using a smaller number of cells (30e6 cells vs. 1e6 cells), which again confirmed that ANBL6 has the highest cell binding percentage and CD46 expression, followed by RPMI8226, MM.1S, and AMO1, with very low binding in the CD46-KO MM.1S cell line (Supplementary Fig. S1). Flow cytometry was also performed to measure CD46 expression on different multiple myeloma cell lines, demonstrating strong surface expression of CD46 in AMO1, ANBL6, MM.1S, and RPMI8226 (Fig. 1C). We used a bioinformatics approach to evaluate the generality of these findings in human multiple myeloma. Leveraging myeloma patient tumor gene expression data in the Multiple Myeloma Research Foundation CoMMpass database (research.themmr.org), we found a range of CD46 expression in primary tumors. Importantly all appeared positive for CD46 ($n = 776$, CoMMpass release IA13; Supplementary Fig. S2A). Given that CD46 surface antigen density may at least partially determine $[^{89}\text{Zr}]\text{DFO-YS5}$ binding, we further performed computational epigenetic analysis to identify possible transcriptional regulators of CD46. We identified transcription factors USF1 and XBP1 as potential positive regulators of CD46, and STAT4 as a potential negative regulator (Supplementary Fig. S2B–S2D). Together, these findings support the notion that CD46 is highly expressed in myeloma and can be recognized by $[^{89}\text{Zr}]\text{Zr-DFO-YS5}$.

$\mu\text{PET/CT}$ imaging and biodistribution analysis of $[^{89}\text{Zr}]\text{Zr-DFO-YS5}$ in subcutaneous MM.1S tumor models reveal high tumor-to-background ratio imaging and CD46-targeted mediated uptake

Next, we evaluated the imaging properties of $[^{89}\text{Zr}]\text{Zr-DFO-YS5}$ in subcutaneous models ($n = 4$) of multiple myeloma using $\mu\text{PET/CT}$. The imaging study showed that $[^{89}\text{Zr}]\text{Zr-DFO-YS5}$ shows high uptake in the tumor and a relatively low background in other organs (Fig. 2A). A series of control experiments were

performed to verify the specificity of $[^{89}\text{Zr}]\text{Zr-DFO-YS5}$ targeting CD46, including a blocking study ($n = 3$; Fig. 2B), imaging with a nonspecific binding ^{89}Zr -labeled antibody $[^{89}\text{Zr}]\text{Zr-DFO-IgG}$ ($n = 4$; Fig. 2C), and imaging in CD46-knockout tumor model ($n = 5$; Fig. 2D). These three control studies showed comparatively low uptake in the tumor. The biodistribution studies showed that the tumor uptake of $[^{89}\text{Zr}]\text{Zr-DFO-YS5}$ was $17.57 \pm 3.23\% \text{ IA/g}$ (Supplementary Tables S1 and S2) at 6 days after injection, significantly higher than the blocking group $8.51 \pm 2.35\% \text{ IA/g}$ ($P < 0.0001$; Supplementary Tables S3 and S4), nonspecific binding ^{89}Zr -labeled antibody group $[^{89}\text{Zr}]\text{Zr-DFO-IgG}$ $4.07 \pm 0.66\% \text{ IA/g}$ ($P < 0.0001$; Supplementary Tables S5 and S6), and CD46-knockout MM.1S group $3.02 \pm 0.85\% \text{ IA/g}$ ($P < 0.0001$; Fig. 2E; Supplementary Tables S7 and S8). These results demonstrated that $[^{89}\text{Zr}]\text{Zr-DFO-YS5}$ can specifically image CD46-positive multiple myeloma with high uptake in the tumor.

$\mu\text{PET/CT}$ imaging and biodistribution analysis using $[^{89}\text{Zr}]\text{Zr-DFO-YS5}$ in subcutaneous RPMI8226 and ANBL6 multiple myeloma models reveals high tumoral uptake

Two additional multiple myeloma cell lines, RPMI8226 and ANBL6, were studied by PET imaging and biodistribution analysis. In RPMI8226 tumor models, PET imaging revealed that mice administered $[^{89}\text{Zr}]\text{Zr-DFO-YS5}$ ($n = 4$) showed high uptake (Fig. 3A). In contrast, mice administered $[^{89}\text{Zr}]\text{Zr-DFO-IgG}$ ($n = 5$) showed low tumor uptake (Fig. 3B). The biodistribution studies were consistent with the imaging results. In the RPMI8226 model, the tumor uptake is $9.65 \pm 1.43\% \text{ IA/g}$ (Fig. 3C; Supplementary Tables S9 and S10), higher than the nonspecific binding group $3.65 \pm 0.77\% \text{ IA/g}$ ($P < 0.0001$; Supplementary Tables S11 and S12). Similarly, in the ANBL6 tumor model, mice administered $[^{89}\text{Zr}]\text{Zr-DFO-YS5}$ ($n = 4$) showed high uptake (Fig. 3D), whereas mice administered $[^{89}\text{Zr}]\text{Zr-DFO-IgG}$ ($n = 5$) showed low tumor uptake (Fig. 3E). In the ANBL6 model, the tumor uptake is $7.47 \pm 0.86\% \text{ IA/g}$ ($n = 4$; Fig. 3F; Supplementary Tables S13 and S14), higher than the control group $3.44 \pm 0.76\% \text{ IA/g}$ ($n = 5$; $P = 0.0005$; Supplementary Tables S15 and S16).

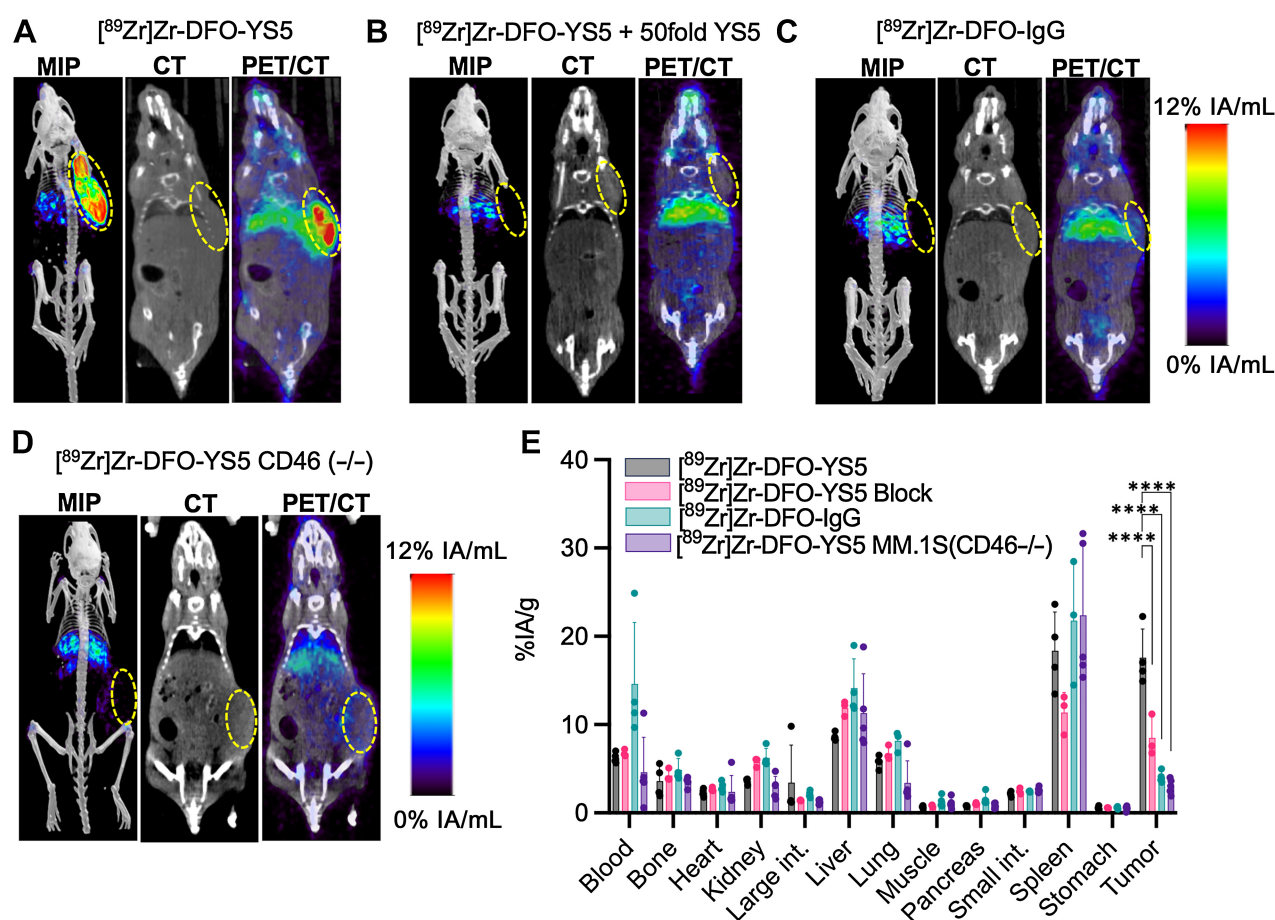


Figure 2.

[⁸⁹Zr]Zr-DFO-YS5 PET/CT can detect subcutaneous MM.1S xenografts *in vivo*. MIP and CT and μ PET/CT fusion images of (A) [⁸⁹Zr]Zr-DFO-YS5 ($n = 4$), (B) 50-fold YS5 + [⁸⁹Zr]Zr-DFO-YS5 ($n = 3$), and (C) [⁸⁹Zr]Zr-DFO-IgG in NSG mice ($n = 4$) bearing subcutaneous MM.1S xenografts at 6 days post-injection. D, MIP and CT and μ PET/CT fusion images of [⁸⁹Zr]Zr-DFO-YS5 in NSG mice bearing ($n = 5$) subcutaneous CD46-KO MM.1S (CD46^{-/-}) xenograft at 6 days post-injection. E, Biodistribution of [⁸⁹Zr]Zr-DFO-YS5, [⁸⁹Zr]Zr-DFO-YS5 + 50-fold YS5, [⁸⁹Zr]Zr-DFO-IgG in NSG mice bearing subcutaneous MM.1S xenograft, and biodistribution of [⁸⁹Zr]Zr-DFO-YS5 in NSG mice bearing subcutaneous CD46-KO MM.1S (CD46^{-/-}) xenograft at 6 days after injection. Two-way ANOVA P values are indicated as *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

μ PET/CT imaging and biodistribution of [¹⁸F]-FDG in subcutaneous MM.1S, RPMI8226 and ANBL6 tumor models

We performed [¹⁸F]-FDG μ PET/CT, which is the standard of care for clinical staging and restaging of multiple myeloma. [¹⁸F]-FDG imaging was performed and it revealed moderate uptake in the tumor in all three animal models ($n = 5$; Fig. 4A–C). The biodistribution study showed the uptake in MM.1S, RPMI8226, and ANBL6 xenografts were $5.76 \pm 1.79\%$ IA/g (Supplementary Tables S17 and S18), $4.90 \pm 0.73\%$ IA/g (Supplementary Tables S19 and S20), and $5.49 \pm 0.70\%$ IA/g (Supplementary Tables S21 and S22), respectively. In the other organs, the uptake in the brain, large intestine, and heart was higher than in the tumor (Fig. 4D).

μ PET/CT imaging and biodistribution analysis of [⁸⁹Zr]Zr-DFO-YS5 and [¹⁸F]-FDG in systemic multiple myeloma models

After demonstrating the imaging characteristics in subcutaneous xenograft models, we moved to evaluate the imaging performance of [⁸⁹Zr]Zr-DFO-YS5 ($n = 5$) in a more clinically relevant systemic model. At 10 days after administration of MM.1S-luc cells, multiple myeloma cells were mainly detected at the bone marrow, in some cases

with a single tumor deposit and other cases with multiple deposits (Fig. 5A). One representative mouse monitored with BLI and [⁸⁹Zr]Zr-DFO-YS5 μ PET/CT demonstrated a high signal in the femur bone marrow close to the knee (Fig. 5A and B). An *ex vivo* PET imaging and BLI study were performed after dissection to compare the uptake in different organs. The *ex vivo* BLI indicated heterogeneous bone marrow tumor involvement, with matching areas of [⁸⁹Zr]Zr-DFO-YS5 uptake ($n = 5$; Fig. 5C). Similarly, one representative mouse from the blocking group was monitored with BLI (Fig. 5D). For the blocking control study, mice ($n = 4$) were administered 50-fold cold YS5 together with [⁸⁹Zr]Zr-DFO-YS5, which reduced the uptake in bone marrow in the femur (Fig. 5E). The biodistribution studies in the systemic model showed that [⁸⁹Zr]Zr-DFO-YS5 had a higher uptake in the bone marrow $7.81 \pm 2.74\%$ IA/g at 4 days (Supplementary Tables S23 and S24) after injection than the blocked group $3.52 \pm 0.96\%$ IA/g (Fig. 5F; Supplementary Tables S25 and S26). Again, one representative mouse was monitored with BLI from the [¹⁸F]-FDG injected cohort (Fig. 5G). [¹⁸F]-FDG PET/CT imaging was performed, and it revealed a moderate uptake in the tumor ($n = 4$; Fig. 5H). The biodistribution study showed a similar pattern to the subcutaneous

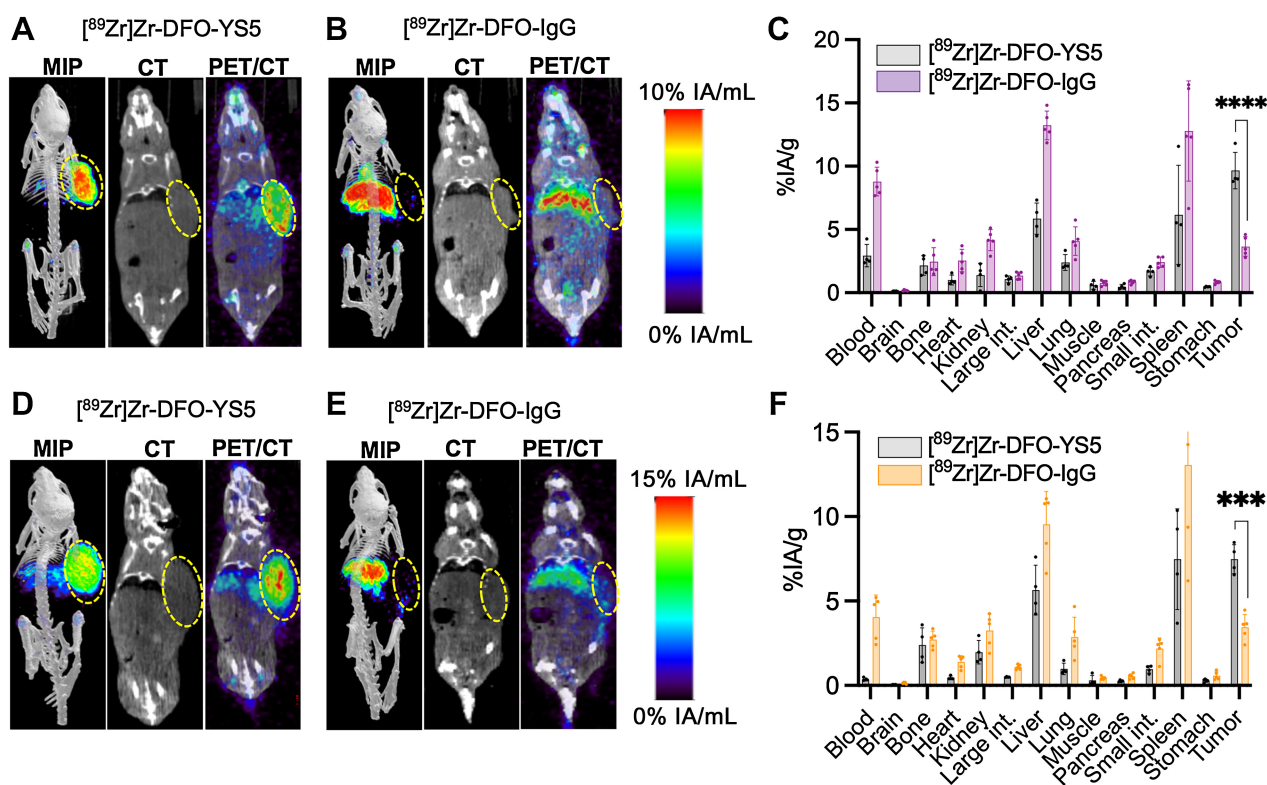


Figure 3.

$[^{89}\text{Zr}]$ Zr-DFO-YS5 imaging and biodistribution in RPMI8226 and ANBL6 subcutaneous xenograft models. MIP and CT and μ PET/CT fusion images of (A) $[^{89}\text{Zr}]$ Zr-DFO-YS5 ($n = 4$) and (B) $[^{89}\text{Zr}]$ Zr-DFO-IgG ($n = 5$) in NSG mice bearing subcutaneous RPMI8226 xenografts at 6 days after injection. C, Biodistribution of $[^{89}\text{Zr}]$ Zr-DFO-YS5 and $[^{89}\text{Zr}]$ Zr-DFO-IgG in NSG mice bearing subcutaneous RPMI8226 xenografts at 6 days after injection. MIP and CT and μ PET/CT fusion images of (D) $[^{89}\text{Zr}]$ Zr-DFO-YS5 ($n = 4$) and (E) $[^{89}\text{Zr}]$ Zr-DFO-IgG ($n = 5$) in NSG mice bearing subcutaneous ANBL6 xenografts at 6 days after injection. F, Biodistribution of $[^{89}\text{Zr}]$ Zr-DFO-YS5 and $[^{89}\text{Zr}]$ Zr-DFO-IgG in NSG mice bearing subcutaneous ANBL6 xenografts at 6 days after injection. Two-way ANOVA P values are indicated as *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

model, in which the brain, heart, and large intestine showed an uptake higher than the tumor (Fig. 5I; Supplementary Tables S27 and S28). These results demonstrate that both $[^{18}\text{F}]$ -FDG PET/CT and $[^{89}\text{Zr}]$ Zr-DFO-YS5 could detect the systemic tumor.

Similarly, the imaging capability of $[^{89}\text{Zr}]$ Zr-DFO-YS5 was demonstrated in the RPMI8226 systemic multiple myeloma model. $[^{18}\text{F}]$ -FDG imaging was also performed in the RPMI8226 systemic model. The BLI (Supplementary Fig. S3A) and μ PET/CT imaging with $[^{89}\text{Zr}]$ Zr-DFO-YS5 revealed high uptake in the femoral bone marrow (Supplementary Fig. S3B). $[^{18}\text{F}]$ -FDG shows moderate uptake (Supplementary Fig. S3C and S3D). *Ex vivo* biodistribution also indicates that $[^{89}\text{Zr}]$ Zr-DFO-YS5 showed high uptake in the bone marrow $11.57 \pm 8.54\%$ IA/g (Supplementary Fig. S3E; Supplementary Tables S29 and S30) and $[^{18}\text{F}]$ -FDG showed $3.26 \pm 2.71\%$ IA/g (Supplementary Fig. S3F; Supplementary Tables S31 and S32).

Radiopharmaceutical therapy with $[^{225}\text{Ac}]$ Ac-DOTA-YS5 in the MM.1S systemic tumor model demonstrates therapeutic efficacy, particularly in fractionated dosing regimens

Before moving on to the treatment study we performed a magnetic bead-based assay to determine the immunoreactivity of $[^{225}\text{Ac}]$ Ac-DOTA-YS5 towards CD46. This study showed that the binding of $[^{225}\text{Ac}]$ Ac-DOTA-YS5 was $91\% \pm 4\%$ demonstrating preservation of immunoreactivity of radiolabeled antibody. The addition of 10-fold excess of cold YS5, and with no CD46 protein, significantly reduced

bead binding to $13.66 \pm 2\%$ and $11.67 \pm 0.5\%$ (Supplementary Fig. S4). A treatment study was designed to evaluate the therapeutic efficacy of $[^{225}\text{Ac}]$ Ac-DOTA-YS5 in the MM.1S systemic tumor model (schematized on Fig. 6A and presented in detail in Materials and Methods). The metastatic tumor study was informed by a pilot treatment study in subcutaneous xenograft mice which demonstrated dose-dependent antitumor efficacy (Supplementary Fig. S5), but also rapid tumor growth (Supplementary Fig. S5A), body weight loss (Supplementary Fig. S5B; particularly at the highest, $0.25 \mu\text{Ci}$ dose level), and mortality in all arms (Supplementary Fig. S5C), suggesting that the more robust and clinically relevant metastatic model would be more suitable to evaluate therapeutic efficacy. In the metastatic tumor study, the control saline arm demonstrated continued tumor progression, with all mice reaching the endpoint at day 38 (Fig. 6B). In contrast, the $[^{225}\text{Ac}]$ Ac-DOTA-YS5 treated groups demonstrated a dose-dependent decrease in tumor burden in both the single and fractionated dosing groups. No significant body weight loss (Fig. 6C) was observed other than one mouse in the $0.125 \mu\text{Ci}$ $[^{225}\text{Ac}]$ Ac-DOTA-YS5 fractionated dose arm, which had to be euthanized at day 110 post-administration due to body weight loss. One unexpected finding was also significant tumor burden reduction (demonstrated by BLI) in the $0.125 \mu\text{Ci}$ dose of $[^{225}\text{Ac}]$ Ac-DOTA-IgG, but also greatly enhanced toxicity as manifested by substantial body weight loss requiring euthanasia in 5 of 7 mice. This correlated with high blood retention and marrow uptake of the nontargeting therapeutic

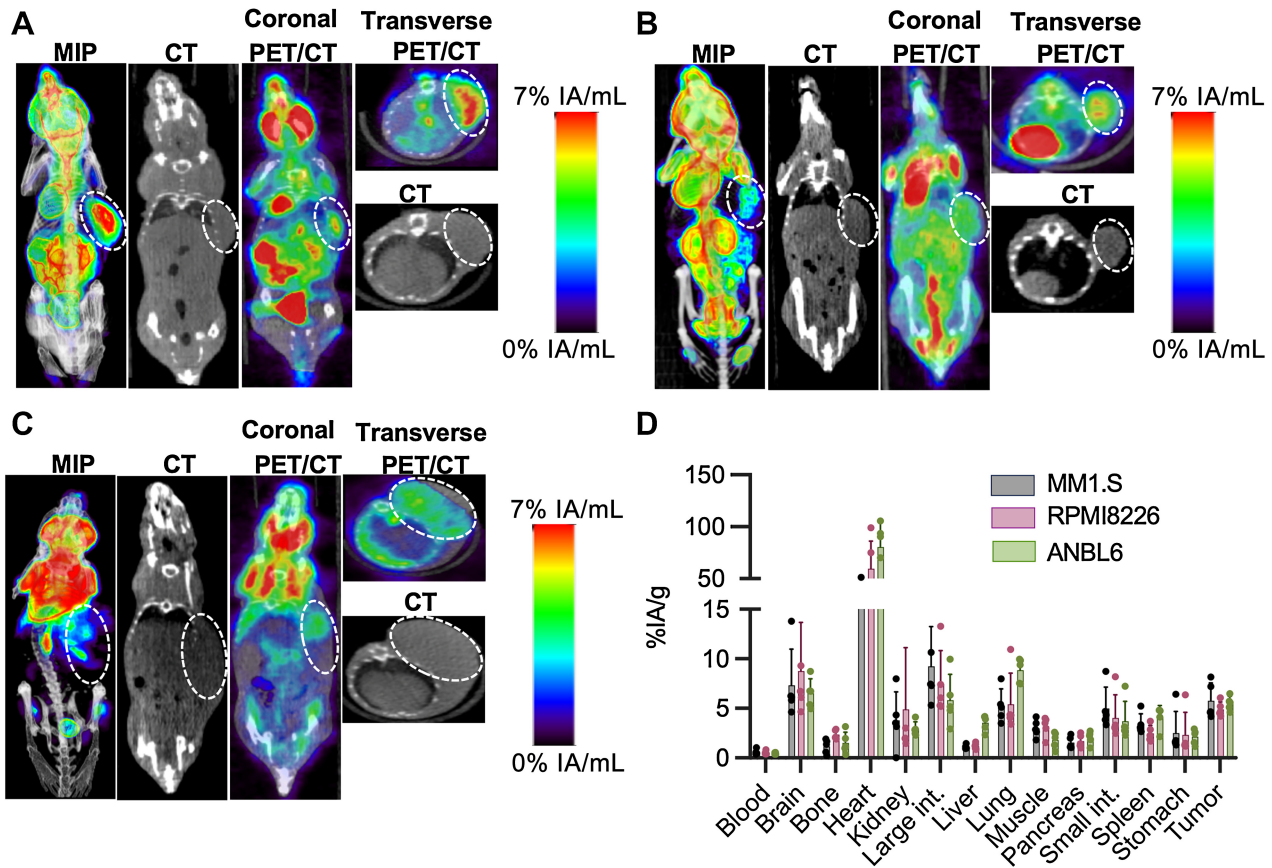


Figure 4. $[^{18}\text{F}]$ -FDG imaging and biodistribution in subcutaneous MM1S, RPMI8226, and ANBL6 xenograft models. MIP and CT and μ PET/CT fusion images of $[^{18}\text{F}]$ -FDG (coronal and transverse posture) in (A) MM1S ($n = 5$), (B) RPMI8226 ($n = 5$), and (C) ANBL6 ($n = 5$) subcutaneous xenografts at 1 hour after injection. D, Biodistribution of $[^{18}\text{F}]$ -FDG in MM1.S, RPMI8226, and ANBL6 subcutaneous xenografts after μ PET/CT imaging at 1 hour after injection.

radiopharmaceutical. This observation is corroborated by BLI (Supplementary Fig. S6A) and PET imaging (Supplementary Fig. S6B), demonstrating relatively high systemic uptake of $[^{89}\text{Zr}]$ Zr-DFO-IgG in the MM1.S systemic model. The biodistribution study (Supplementary Fig. S6C) also confirmed the nonspecific uptake of $[^{89}\text{Zr}]$ Zr-DFO-IgG in the MM1.S systemic model (Supplementary Tables S33 and S34). To confirm the nonspecific uptake of $[^{89}\text{Zr}]$ Zr-DFO-IgG, we performed μ PET/CT imaging and a biodistribution study with healthy nude mice and NSG healthy mice. The PET imaging (Supplementary Fig. S7A and S7B) and biodistribution (Supplementary Fig. S7C) showed high diffuse nonspecific uptake of $[^{89}\text{Zr}]$ Zr-DFO-IgG in the bone marrow, spine, and spleen in NSG mice, which confirms that the accumulation of $[^{89}\text{Zr}]$ Zr-DFO-IgG is strain-specific. These findings likely explain the therapeutic effect and weight loss in the cohort of mice injected with $[^{225}\text{Ac}]$ Ac-DOTA-IgG.

The Kaplan–Meier curve showed that the median survival time of mice in the vehicle group was 38 days, whereas the median survival of the 0.0625 μCi and 0.125 μCi $[^{225}\text{Ac}]$ Ac-DOTA-YS5-treated group was 59 days and 94 days, ($P < 0.05$) and ($P < 0.05$), respectively, and with three fractions of 0.0625 μCi each, the median survival was 91 ($P < 0.001$) and with three fractions of 0.125 μCi each the median survival was not defined (since 4 mice of 7 were still alive) during the end day of the treatment. In addition, the median survival with a 0.125 μCi dose of $[^{225}\text{Ac}]$ Ac-DOTA-IgG was 45 days ($P = 0.25$;

Fig. 6D). These data suggest that a fractionated regimen represents a promising method for treatment.

In addition, comparison with the saline group (Fig. 6E), 0.125 μCi $[^{225}\text{Ac}]$ Ac-DOTA-YS5 injected group shows a decrease in CD138 expression in the femurs (BLI images of the corresponding mice Supplementary Fig. S8) 15 days after injection (Fig. 6F), which confirms tumor-targeted cell death and reduction in tumor burden.

The treatment study was completed at day 120 with four mice remaining in the 0.125 μCi fractionated dosing group. $[^{89}\text{Zr}]$ Zr-DFO-YS5 PET/CT imaging of all surviving mice from the 0.125 μCi fractionated dose group at day 120 indicated that one mouse had tumor detected with BLI (Supplementary Fig. S9A) and $[^{89}\text{Zr}]$ Zr-DFO-YS5 PET/CT imaging (Supplementary Fig. S9B), whereas one mouse had a focus of $[^{89}\text{Zr}]$ Zr-DFO-YS5 PET/CT imaging found in the shoulder, with no matched BLI signal, which could represent an additional lesion (Supplementary Fig. S9C and S9D). In addition, two mice had no detectable tumor by either BLI or $[^{89}\text{Zr}]$ Zr-DFO-YS5 PET/CT (Supplementary Fig. S9E, S9F, S9G, and S9H). These images suggest a potential for using $[^{89}\text{Zr}]$ Zr-DFO-YS5 PET/CT to monitor the therapeutic effect of $[^{225}\text{Ac}]$ Ac-DOTA-YS5, although these studies are limited by single time point analysis. Moreover, they do not support the loss of CD46 expression as a resistance mechanism following treatment in the mice with residual tumor. Histologic analysis of recovered tissues from this cohort demonstrated no detectable damage

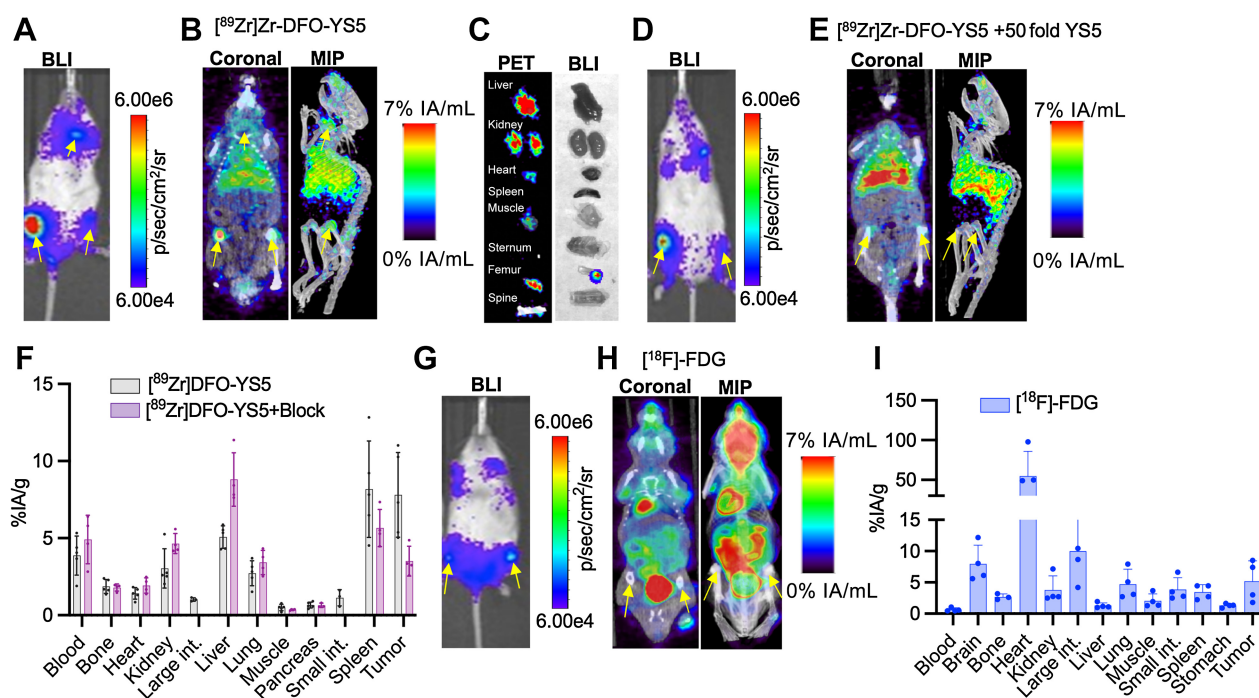


Figure 5.

$[^{89}\text{Zr}]\text{Zr-DFO-YS5}$ demonstrates favorable PET imaging characteristics in the MM.1S systemic tumor model. **A**, BLI of NSG mice bearing MM.1S systemic model revealed tumor was located in the femoral bone marrow. Sites of disease involvement are indicated with an arrow. **B**, Maximum intensity projections (MIP) and $\mu\text{PET}/\text{CT}$ fusion images of $[^{89}\text{Zr}]\text{Zr-DFO-YS5}$ in NSG mice ($n = 5$) bearing systemic MM.1S xenograft at 4 days after injection revealed high tumor uptake in femur bone marrow, indicated by arrows. **C**, Correlation of *ex vivo* $[^{89}\text{Zr}]\text{Zr-DFO-YS5}$ PET images with bioluminescence, demonstrating matching regions of osseous PET uptake with tumor BLI signal. **D**, BLI of NSG mice bearing MM.1S systemic model. Sites of disease involvement are indicated with an arrow. **E**, MIP and $\mu\text{PET}/\text{CT}$ of $[^{89}\text{Zr}]\text{Zr-DFO-YS5}$ with 50-fold block of cold YS5 in NSG mice ($n = 4$) bearing MM.1S systemic xenograft at 4 days after injection, demonstrating no detectable tumor uptake above background at the documented sites of tumor involvement (arrows). **F**, Biodistribution of $[^{89}\text{Zr}]\text{Zr-DFO-YS5}$, $[^{89}\text{Zr}]\text{Zr-DFO-YS5}$ with 50-fold block of cold YS5 in NSG mice bearing systemic MM.1S xenograft at 4 days after injection. **G**, BLI of NSG mice bearing MM.1S systemic model. Sites of disease involvement are indicated with an arrow. **H**, MIP and $\mu\text{PET}/\text{CT}$ fusion images of $[^{18}\text{F}]\text{-FDG}$ in NSG mice ($n = 4$) bearing MM.1S systemic xenograft at 1 hour after injection, demonstrating moderate tumor uptake. **I**, Biodistribution of $[^{18}\text{F}]\text{-FDG}$ in NSG mice bearing systemic MM.1S xenograft.

to the liver, kidneys, bone marrow, heart, and spleen (Supplementary Fig. S10). Taken together, these demonstrate optimal outcomes at the fractionated dose level of $0.125 \mu\text{Ci}$ of $[^{225}\text{Ac}]\text{Ac-DOTA-YS5}$ when compared against other arms.

Discussion

In this study, we have presented the systematic evaluation of CD46-directed PET imaging with $[^{89}\text{Zr}]\text{Zr-DFO-YS5}$ in a panel of preclinical models of multiple myeloma. Notably, high uptake was seen in all tested models including MM.1S, RPMI8226, and ANBL6. Relevant control studies including blocking, nonspecific IgG imaging, and CD46 knockout models demonstrate that the uptake of this agent is specific to the CD46 target, and not dominated by nonspecific accumulation. The apparent discrepancy in %ID/g when rank ordering ANBL6, RPMI8226, and MM.1S *in vitro* and *in vivo* may be due, at least in part, to the differences in the size of the tumors, as the total %ID per tumor (Supplementary Fig. S11) was $11.85 \pm 1.06\%$, $8.38 \pm 2.36\%$, $5.00 \pm 0.31\%$ for these cell lines, respectively. Additional features that could cause a discrepancy between the *in vitro* and *in vivo* binding and uptake of these radiopharmaceuticals could be variability in vascularity, tumor necrosis, or other features governing the enhanced permeability and retention effect. Our results demonstrated that $[^{89}\text{Zr}]\text{Zr-DFO-YS5}$ could effectively detect subcutaneous as well as

systemic multiple myeloma tumors, although these results should be interpreted with caution as YS5 does not bind to endogenous mouse CD46. Taken together, these results support the further development of CD46 PET imaging with $[^{89}\text{Zr}]\text{Zr-DFO-YS5}$, and indeed we have recently opened a clinical multiple myeloma PET imaging trial (NCT05892393) using this agent, similar to our ongoing prostate cancer PET imaging clinical trial (NCT05245006).

Clinical translation of this PET agent is encouraged by important limitations in the existing methods for imaging multiple myeloma. Whole-body low-dose CT is preferred for detecting lytic bone lesions, and MRI is the method of choice for detecting bone marrow involvement in multiple myeloma (38). In addition, $[^{18}\text{F}]\text{-FDG}$ PET/CT is considered the preferred imaging method to detect extramedullary multiple myeloma lesions and monitor metabolic treatment response to therapy. However, lesions with low metabolic rates may be undetectable with $[^{18}\text{F}]\text{-FDG}$ (39, 40). In addition to $[^{18}\text{F}]\text{-FDG}$, new PET tracers such as L-methyl- $[^{11}\text{C}]\text{-methionine}$ (MET) have been emerging as an imaging agent that targets different metabolic pathways (41).

We propose that CD46-directed PET imaging with $[^{89}\text{Zr}]\text{Zr-DFO-YS5}$ demonstrates promise for clinical evaluation, as currently being investigated for other tracers such as those directed against CD38, CXCR4, and others (42–45). A potential advantage of $[^{89}\text{Zr}]\text{Zr-DFO-YS5}$ compared with previously published agents is the tumor-specific CD46 epitope recognized by YS5 (27); all other explored targets are

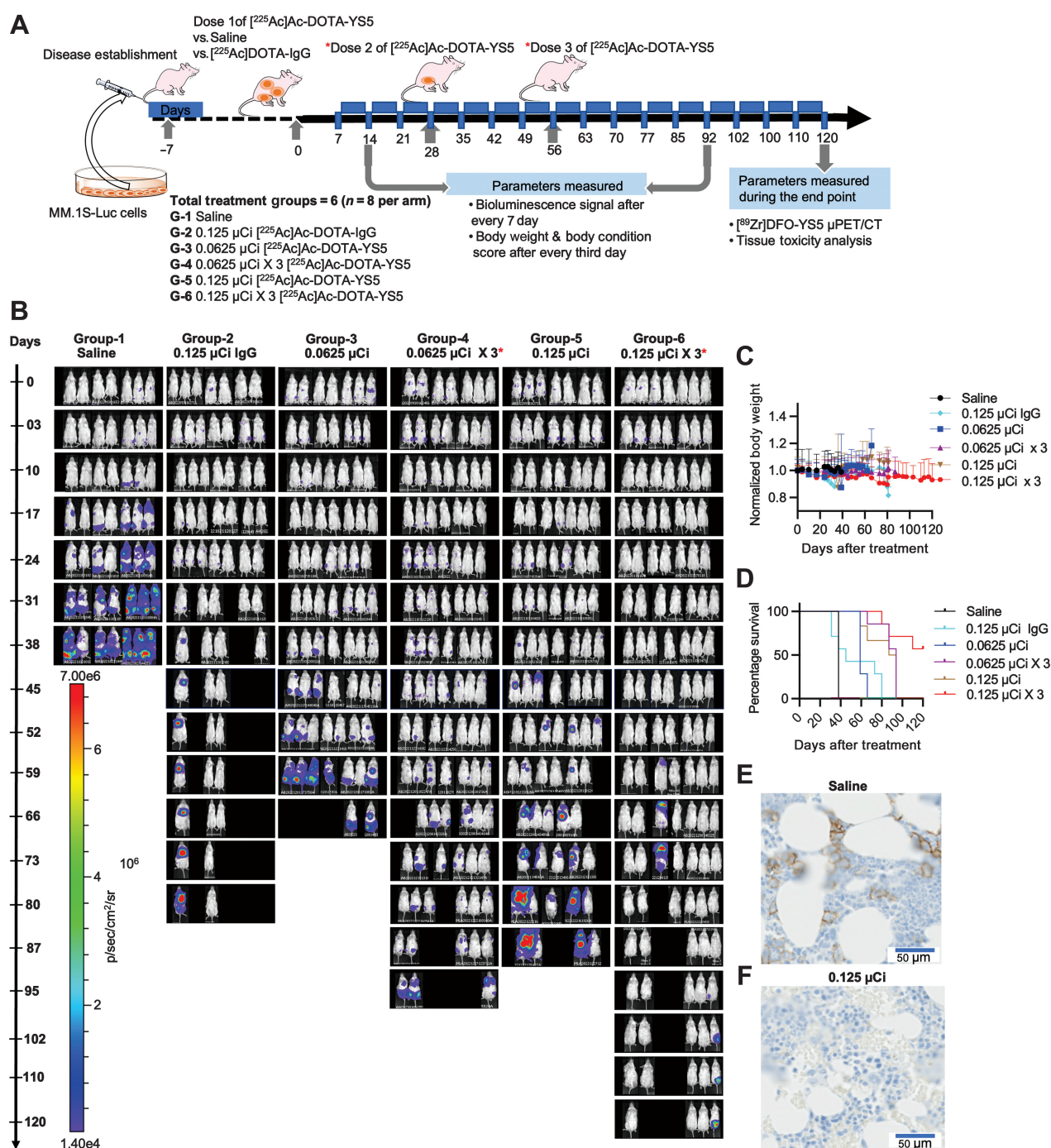


Figure 6.

$[^{225}\text{Ac}]\text{Ac-DOTA-YS5}$ is an effective treatment for multiple myeloma in the MM.1S metastatic model. **A**, Schematic for therapeutic study. **B**, Serial BLI imaging indicates reduced tumor burden in the treatment groups (ventral view; $n = 8$). **C**, Body weight measurements in control and treatment groups. **D**, Kaplan-Meier curve demonstrates dose-dependent improvement in overall survival in the treatment arms. **E**, CD138 expressing cells in femurs in mice from the control group ($n = 1$) treated with saline after 15 days (**F**) decrease in CD138 expressing cells in femurs in mice treated with 0.125 μCi ($n = 1$) of $[^{225}\text{Ac}]\text{Ac-DOTA-YS5}$ after 15 days, confirming tumor-targeted cell death and reduction in tumor burden.

expressed widely on other cell types in addition to multiple myeloma plasma cells, giving scope for false-positive results (46). In addition, CD38 antigen density can be significantly decreased in patients who previously received anti-CD38 mAb therapy (47), a standard part of

current myeloma regimens (48, 49). Given the important limitations of the standard of care methods outlined above, there is great promise for the development of new molecular imaging and theranostic technologies such as $[^{89}\text{Zr}]\text{Zr-DFO-YS5}$ PET in multiple myeloma. Our

imaging results demonstrate the feasibility of clinical translation of CD46-directed PET imaging in patients with multiple myeloma.

In multiple myeloma, radiopharmaceutical therapies demonstrate significant promise. In particular, several ongoing clinical studies are investigating the efficacy of ^{225}Ac -radioimmunoconjugates in a variety of cancers, including prostate cancer and acute myeloid leukemia (50–54). Both clinical and preclinical studies have shown efficacious results for daratumumab, which targets the CD38 protein in multiple myeloma cells. However, not all patients respond to daratumumab therapy (55). One limitation of current radioligand therapy strategies in multiple myeloma is the associated damage to the bone marrow and the need for associated stem cell transplantation. Because CD46 has low expression on normal marrow progenitor cells, targeting this antigen may avoid this complication in clinical studies. In addition, alpha particle-based radioligand therapy can kill malignant bone marrow cells with high-energy linear transfer and minimal effect on normal BM cells (56). Therefore, CD46-targeted radiopharmaceutical therapy with ^{225}Ac Ac-DOTA-YS5 has the potential for reduced bone marrow toxicity in future clinical study.

Recently, Dawickki and colleagues conjugated daratumumab with ^{225}Ac and demonstrated a strong antitumor effect of ^{225}Ac Ac-daratumumab against CD38-positive multiple myeloma tumors and clinical trial for this agent has been opened (NCT053631111; ref. 16). Similarly, treatment with ^{225}Ac Ac-DOTA-YS5 demonstrated favorable outcomes in the MM.1S model. In particular, a fractionated dosing scheme using three fractions consisting of 0.125 μCi each substantially prolonged overall survival and reduced tumor burden compared with other groups (Fig. 6). Poor treatment outcomes were observed during treatment with ^{225}Ac Ac-DOTA-IgG, which is due to nonspecific uptake of the radiotracer in the bone marrow which leads to toxicity (more than 20% body weight loss in most of the mice of this cohort). These data suggest that CD46-targeted radioligand therapy using ^{225}Ac Ac-DOTA-YS5 is a promising strategy for the treatment of multiple myeloma. This is further supported by encouraging results from the phase I trial of CD46 ADC, and FOR46, in relapsed/refractory patients with multiple myeloma. In this study, patients had an overall response rate of 33% among 9 patients treated at dose levels ≥ 1.8 mg/kg (28).

There are important limitations related to this study which merit discussion. First, the mouse models employed in this study do not express human CD46, therefore potentially underestimating background radiopharmaceutical distribution. However, normal tissue IHC data demonstrate relatively low YS5 binding in normal human tissues expressing CD46 (26, 57), at least partially mitigating this concern. Another limitation of our study is that we have not performed IHC in the subcutaneous tumor sections (MM.1S, ANBL6, and RPMI8226), which could provide additional information about CD46 expression in different tumor models. In addition, our ^{225}Ac treatment studies were performed in the NSG mouse model due to our prior experience in growing the sensitive multiple myeloma cells in this strain. However, it is well known that NSG mice are more sensitive to radiation due to their highly suppressed DNA damage repair (58). Therefore, the toxicity of the therapy may be accentuated compared with other mouse models, potentially reducing the therapeutic efficacy and overall survival results compared with which might be obtained otherwise. However, this criticism is tempered by the promising overall therapeutic results in the fractionated regimen group, as well as the relative lack of toxicity observed. Finally, the ^{225}Ac Ac-DOTA-YS5 agent utilized in this study requires a relatively high chelator-to-antibody ratio, potentially blunting therapeutic efficacy. This final

limitation could be addressed by using more effective chelators such as Macropa, now underway in our laboratories (59, 60).

This study builds upon prior and ongoing work in our laboratories on the development of CD46 theranostics. As CD46 is a cancer antigen, which is widely expressed in many other malignancies, this suggests the potential for CD46 PET imaging and radiopharmaceutical therapy not only in multiple myeloma but also in other malignancies. This possibility will be tested in our clinical trial of ^{89}Zr Zr-DFO-YS5 PET imaging in patients with multiple myeloma.

Conclusion

In this study, “ ^{89}Zr Zr-DFO-YS5 was tested as a CD46-targeted immunoPET imaging probe for MM” in different murine models, with higher tumor uptake and lower background than ^{18}F -FDG. A YS5-based therapeutic agent, ^{225}Ac Ac-DOTA-YS5, was also successfully prepared, and demonstrated effective antitumor treatment. Overall, ^{89}Zr Zr-DFO-YS5 shows great potential for clinical translation as an imaging agent and theranostic companion biomarker, and ^{225}Ac Ac-DOTA-YS5 shows promising results for further development as a multiple myeloma treatment.

Authors' Disclosures

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Authors' Contributions

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Note

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