In Vitro and In Vivo Comparison of 3,2-HOPO Versus Deferoxamine-Based Chelation of Zirconium-89 to the Antimesothelin Antibody Anetumab

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Abstract

Introduction: [227Th]Th-3,2-HOPO-MSLN-mAb, a mesothelin (MSLN)-targeted thorium-227 therapeutic conjugate, is currently in phase I clinical trial; however, direct PET imaging using this conjugate is technically challenging. Thus, using the same MSLN antibody, we synthesized 3,2-HOPO and deferoxamine (DFO)-based zirconium-89 antibody conjugates, [89Zr]Zr-3,2-HOPO-MSLN-mAb and [89Zr]Zr-DFO-MSLN-mAb, respectively, and compared them in vitro and in vivo.

Methods: [89Zr]Zr-3,2-HOPO-MSLN-mAb and [89Zr]Zr-DFO-MSLN-mAb were evaluated in vitro to determine binding affinity and immunoactivity in HT29-MSLN and PDX (NCI-Meso16, NCI-Meso21) cells. For both the zirconium-89 conjugates, in vivo studies (biodistribution/imaging) were performed at days 1, 3, and 6, from which tissue uptake was determined.

Results: Both the conjugates demonstrated a low nanomolar binding affinity for MSLN and >95% immunoactivity. In all the three tumor types, biodistribution of [89Zr]Zr-DFO-MSLN-mAb resulted in higher tumor uptake (15.88-28-33% ID/g) at all time points compared with [89Zr]Zr-3,2-HOPO-MSLN-mAb (7–13.07% ID/g). [89Zr]Zr-3,2-HOPO-MSLN-mAb femur uptake was always higher than [89Zr]Zr-DFO-MSLN-mAb, and imaging results concurred with the biodistribution studies.

Conclusions: Even though the conjugates exhibited a high binding affinity for MSLN, [89Zr]Zr-DFO-MSLN-mAb showed a higher tumor and lower femur uptake than [89Zr]Zr-3,2-HOPO-MSLN-mAb. Nevertheless, [89Zr]Zr-3,2-HOPO-MSLN-mAb could be used to study organ distribution and lesion uptake with the caveat of detecting MSLN-positive bone lesions. Clinical trial (NCT03507452).

Keywords: 32-HOPO, antibody conjugate, DFO, mesothelin, PET imaging, zirconium-89

Introduction

Targeted alpha therapy (TAT) is a promising new cancer therapy, which induces DNA double-stranded breaks in cancer cells, by specific delivery of alpha particle emitting radionuclide-labeled tracers to tumors.1–6 At present, several α-particle emitting radionuclides with suitable half-lives are under evaluation for TATs.5,7–9 Thorium-227 has been evaluated to develop TAT for treating various cancers.9–15 Targeted thorium-227 conjugates (TTCs) have demonstrated promising preclinical therapeutic results in acute myeloid leukemia, ovarian, breast, pancreatic, lung, and renal cell carcinoma xenografts.10–13,15 Mesothelin (MSLN) antibody-targeted thorium-227 conjugate (MSLN-TTC; 227Th-anetumab corixetan) has been developed for treating MSLN-positive cancers. MSLN is a...
GPI-anchored glycoprotein known to be overexpressed in malignant mesothelioma, lung, ovarian, pancreatic, breast, gastric, head and neck cancers with restricted expression in healthy tissues.\textsuperscript{16–19} MSLN-TTC was developed by covalently conjugating a fully humanized MSLN-mAb (anetumab, BAY861903) to 3-hydroxypyridin-2-one (3,2-HOPO) chelator and radiolabeling it with thorium-227 to produce \([227\text{Th}]\text{Th}-3,2\text{-HOPO-MSLN-mAb (BAY 2287411)}\).\textsuperscript{11,20} In vivo \([227\text{Th}]\text{Th}-3,2\text{-HOPO-MSLN-mAb has demonstrated therapeutic efficacy in various cancers.}\textsuperscript{9–14} In a study, BAY2287411 in combination with ATR (BAY1895344) and PARP (Olaparib/AZD2281) inhibitors demonstrated synergistic antitumor effects in ovarian cancer xenografts.\textsuperscript{10} Currently, \([227\text{Th}]\text{Th}-3,2\text{-HOPO-MSLN-mAb is under evaluation for safety and tolerability in a multicenter phase I clinical trial (NCT03507452).}\)

Due to the low \(\gamma\)-emission and low abundance of measurable photons in the decay chain of thorium-227, direct imaging using \([227\text{Th}]\text{Th}-3,2\text{-HOPO-MSLN-mAb is technically challenging. However, 3,2-HOPO chelator has been shown to form thermodynamically stable complexes with antheric antitumor effects in ovarian cancer xenografts.}\textsuperscript{10} Thus, the same 3,2-HOPO-MSLN-mAb can be radiolabeled with thorium-227 for therapeutic or zirconium-89 for PET imaging agents. Zirconium-89 based conjugate may enable identification of MSLN-positive tumor lesions as well as study biodistribution of the MSLN antibody conjugate.

Even though there are several other chelators that can be used with zirconium-89, deferoxamine (DFO) is one of the most widely used chelators of zirconium-89 that is currently used for PET imaging in both preclinical and clinical settings.\textsuperscript{24–32} Therefore, in the present work, we synthesized \([89\text{Zr}]\text{Zr-HOPO-MSLN-mAb and [89Zr]Zr-DFO-MSLN-mAb, and evaluated both the conjugates in vitro and in vivo in three different tumor xenografts. In vitro serum stability of these conjugates was also determined. The results of this study may help evaluate [89Zr]Zr-3,2-HOPO-MSLN-mAb and [89Zr]Zr-DFO-MSLN-mAb as PET imaging agents.}

**Methods**

All studies were done on protocols approved by the NIH Animal Care and Use Committee.

**Synthesis of \([89\text{Zr}]\text{Zr-3,2-HOPO-MSLN-mAb}**

A mixture of zirconium-89 (IV) (\(~37\text{MBq, \sim 1 mCi}\)) and 3,2-HOPO-MSLN-mAb (65 \(\mu\text{g}\)) was incubated for 1 h at room temperature and purified by PD-10 column to obtain \([89\text{Zr}]\text{Zr-3,2-HOPO-MSLN-mAb. A detailed description of the radiolabeling procedure and in vitro serum stabilities is provided in the Supplementary Data.}

**Synthesis of \([89\text{Zr}]\text{Zr-DFO-MSLN-mAb}**

The DFO-MSLN-mAb conjugate was prepared following a published literature method using a threefold molar excess of chelator (DFO-Bz-NCS)\textsuperscript{33} with respect to MSLN-mAb. The chelators per antibody ratio (CAR, 0.8) was determined by radiometric isotope dilution assay.\textsuperscript{34} \([89\text{Zr}]\text{Zr-DFO-MSLN-mAb was prepared according to the literature method using \sim 74 \text{MBq (\sim 2mCi) zirconium-89 (IV) and 300 \(\mu\text{g} DFO-MSLN-mAb.}\textsuperscript{26,33}

In vitro saturation and competition assays

For saturation studies, NCI-Meso16, NCI-Meso21, and HT29-MSLN cells were aliquoted at a constant concentration (250,000–500,000 cells/tube) into tubes containing six concentrations of radiolabeled antibody \([89\text{Zr}]\text{Zr-DFO-MSLN-mAb (0.4–16 \text{nM}) or [89Zr]Zr-3,2-HOPO-MSLN-mAb (1–35 \text{nM})}; \text{non-specific binding was determined in the presence of 1 \mu M concentration of unlabeled MSLN-mAb by adding the cells to the same concentrations ([89Zr]Zr-DFO-MSLN-mAb:0.4–16 \text{nM}; [89Zr]Zr-3,2-HOPO-MSLN-mAb:1–35 \text{nM}) of radiolabeled MSLN-mAb conjugates.}

For the competition assay, the same number of cells was added to each tube and incubated with a constant concentration of \([89\text{Zr}]\text{Zr-DFO-MSLN-mAb or [89Zr]Zr-3,2-HOPO-MSLN-mAb in the presence of various concentrations of the unlabeled MSLN-mAb (10–6–10–12 M). After 2h incubation on ice, the cells from both saturation and competition assays were centrifuged and washed twice with cold PBS to separate bound radiolabeled MSLN-Ab conjugate from unbound. Cell bound radioactivity in the cell pellet was determined by \(\gamma\)-counting (PerkinElmer 2480 Wizard3).

The \(K_d\) and \(B_{\max}\) were determined from nonlinear regression curve fit (one-site binding hyperbola, GraphPad Prism 7) from six different concentrations of \([89\text{Zr}]\text{Zr-DFO-MSLN-mAb or [89Zr]Zr-3,2-HOPO-MSLN-mAb. The immunoreactive fraction of the radiolabeled antibodies [89Zr]Zr-DFO-MSLN-mAb or [89Zr]-3,2-HOPO-MSLN-mAb were determined by the Morris method as previously described.}\textsuperscript{35} In brief, the immunoreactivity was derived from saturation and self-displacement assay data.\textsuperscript{33}

**Mouse tumor models**

Athymic female nude mice (NCr-nu/nu, 4- to 6-week old, Charles River) were inoculated in the right shoulder by subcutaneous injection of NCI-Meso16 (\(8 \times 10^6\) cells), NCI-Meso21 (\(10 \times 10^6\) cells), or HT29-MSLN (\(2 \times 10^6\) cells) cancer cells in RPMI:Matrigel (50:50).\textsuperscript{36} Mice were housed under 12:12 light/day cycles, and given standard rodent chow and water ad libitum. All the studies and experiments were performed following approved protocols by NIH ACUC.

**Biodistribution studies**

Tumor-bearing mice (NCI-Meso16, NCI-Meso21, or HT29-MSLN; 200–400 mm\(^3\)) were injected 1 d before radiolabeled MSLN-mAb conjugate injections with irrelevant IgG2a antibody (200 \mu g; Sigma) to block the unspecific accumulation of the MSLN-mAb in the spleen.\textsuperscript{37} Mice were intravenously injected with 1.85 MBq (50 \mu Ci) of either \([89\text{Zr}]\text{Zr-3,2-HOPO-MSLN-mAb or [89Zr]Zr-DFO-MSLN-mAb and euthanized (CO \(_2\) asphyxiation) at days 1, 3, and 6. Blood and tissue samples were collected and weighed, and associated radioactivity (counts per minute, CPM) was determined using a \(\gamma\)-counter (Perkin Elmer 2480 Wizard3). The data were calculated as % injected dose per gram (%ID/g) of the tissue normalized to 20 g mice, tissue:blood ratios, tissue:muscle ratios using the following formula:
%ID/g normalized to 20 g mice =  
\[
\frac{\text{CPM}_{\text{tissue}} \times \text{body weight}(g) \times 100}{\text{tissue weight}(g) \times \text{CPM}_{\text{injected dose}} \times 20(g)}
\]

Tissue: Blood Ratios = \(\frac{\text{(\% Injected dose per gram)}_{\text{tissue}}}{\text{(\% Injected dose per gram)}_{\text{blood}}}\)

Tissue: Muscle Ratios = \(\frac{\text{(\% Injected dose per gram)}_{\text{tissue}}}{\text{(\% Injected dose per gram)}_{\text{muscle}}}\)

For the blocking study, a separate group of HT29-MSLN tumor-bearing mice were injected with \(^{89}\text{Zr}\text{-Zr-3,2-HOPO-MSLN-mAb}\) (1.85 MBq, 50 μCi, 4.5 μg) in the presence or absence of an excess of the unlabeled 3,2-HOPO-MSLN-mAb. HT29-MSLN tumor-bearing mice were anesthetized using isoflurane/O₂ (1.5–3%v/v) and imaged at 1, 3, or 6 d. NCI-Meso16 and NCI-Meso21 mouse xenografts were only imaged 3 d postinjection of the radioactive conjugates. Whole-body static images were obtained using the BioPET scanner, following these parameters: 2 bed positions, FOV = 2.0 cm, image time = 10. CT scans of the mice were obtained by using the BioPET scanner, following these parameters: X-ray = 180, voltage (kV) = 50, # projection = 360. The PET/CT images were reconstructed and coregistered to present the data.

Data analysis

Data analysis for the in vitro assay was performed using GraphPad Prism 7 and TableCurve2D v5.01. In vivo PET/CT images were reconstructed using MIMO software. A significance test was performed using the Student t-test.

Results

Conjugation, radiochemistry, and serum stability

The initial radiolabeling of the 3,2-HOPO-MSLN-mAb conjugate (Fig. 1A) was conducted according to the literature method reported for the zirconium-89 labeling of DFO using \(~1\) mCi of zirconium-89 and 130 μg of 3,2-HOPO-MSLN-mAb in a total volume of 0.25 mL.\(^{33}\) A high radiochemical yield (80%–85%) was observed; however, the amount of aggregate (30%) was also high. To minimize the amount of aggregate variable ratio of zirconium-89 to 3,2-HOPO-MSLN-mAb was tested. The results indicated that optimum radiochemical yield was obtained when \(~1\) mCi of zirconium-89 was incubated with 65 μg of 3,2-HOPO-MSLN-mAb in a total volume of 1 mL.

DFO-MSLN-mAb conjugate (Fig. 1B) was radiolabeled with zirconium-89 to prepare \(^{89}\text{Zr\text{-Zr-DFO-MSLN-mAb}}\). \(^{33}\) The isolated radiochemical yields were in the range of 52%–76% \((n = 20)\) for \(^{89}\text{Zr}\text{-Zr-3,2-HOPO-MSLN-mAb}\) and 90%–92% \((n = 8)\) for \(^{89}\text{Zr}\text{-DFO-MSLN-mAb}\). The molar activities of the conjugates were 22,200–77,700 MBq/μmol (600–2100 μCi/μmol) with 82%–95% radiochemical purity (Fig. 1C, D). HPLC chromatogram (size exclusion) at 280 nm indicated the presence of 5%–18% aggregation.

In vitro serum stability of the conjugates was tested in a whole human serum over 4 d (Supplementary Table S1) at 37°C. The SE-HPLC chromatogram revealed the gradual decomposition of both the radiolabeled conjugates. No significant differences in the amount of intact conjugates were observed between the two conjugates on days 1 and 2 (Supplementary Figure S1 and S2). However, by day 4, the amount of intact \(^{89}\text{Zr\text{-Zr-DFO-MSLN-mAb}}\) (46%) was twice the amount of intact \(^{89}\text{Zr\text{-3,2-HOPO-MSLN-mAb}}\) (23%). The in vitro serum stability study indicated slower decomposition of \(^{89}\text{Zr\text{-DFO-MSLN-mAb}}\) after 2 d of incubation at 37°C. HPLC chromatogram indicated that the major decomposition product \((\sim 12\text{ min})\) of \(^{89}\text{Zr\text{-3,2-HOPO-MSLN-mAb}}\) is different from the major decomposition product \((\sim 15\text{ min})\) of \(^{89}\text{Zr\text{-DFO-MSLN-mAb}}\). However, no attempts were made to characterize those decomposition products.

In vitro binding studies

Compared with NCI-Meso21 (34 × 10³ MSLN per cell) and HT29-MSLN cells (249 × 10³ MSLN per cell), NCI-Meso16 cells (178.41 × 10³ MSLN per cell) expressed relatively low levels of MSLN (Fig. 3A). In NCI-Meso16, NCI-Meso21, and HT29-MSLN cells, \(^{89}\text{Zr\text{-DFO-MSLN-mAb}}\) exhibited \(K_d\) of 0.16 ± 0.02 nM, 0.29 ± 0.02 nM, and 0.59 ± 0.06 nM, respectively, for MSLN (Figs. 2 and 3B). In the same cancer cell lines, the binding affinity of \(^{89}\text{Zr\text{-3,2-HOPO-MSLN-mAb}}\) for MSLN was found to be 2.1 ± 0.09 nM (NCI-Meso16), 2.3 ± 0.46 nM (NCI-Meso21), and 1.9 ± 0.39 nM (HT29-MSLN; Figs. 2 and 3B). Even though both the conjugates exhibited high binding affinity, the affinity of \(^{89}\text{Zr\text{-DFO-MSLN-mAb}}\) for MSLN was significantly higher \((p < 0.05)\) than \(^{89}\text{Zr\text{-3,2-HOPO-MSLN-mAb}}\). In all the three cell lines, uptake of both the radiolabeled MSLN-mAb conjugates could be blocked in the presence of unlabeled MSLN-mAb, indicating MSLN-mediated uptake of the conjugates (Fig. 2). The immunoreactivity for \(^{89}\text{Zr\text{-DFO-MSLN-mAb}}\) and \(^{89}\text{Zr\text{-3,2-HOPO-MSLN-mAb}}\) was determined to be 95% and 96%, respectively (Fig. 4).

In vivo biodistribution and imaging studies

Pharmacokinetics of \(^{89}\text{Zr\text{-3,2-HOPO-MSLN-mAb}}\) were determined in HT29-MSLN xenografts for 6 d (Fig. 5A). The uptake of \(^{89}\text{Zr\text{-3,2-HOPO-MSLN-mAb}}\) in nontarget tissues either declined or remained the same over the study period except for the femur. Among the nontarget tissues, the highest uptake was observed in the femur, which significantly increased (day 1: 6.74%ID/g; day 3: 16.52%ID/g; day 6: 15.40%ID/g) over the 6 d (Fig. 5A). Radioactive accumulation in the femur may likely indicate loss of free zirconium-89 from the conjugate, which can be scavenged by the bone.\(^{38,39}\)

\(^{89}\text{Zr\text{-Zr-3,2-HOPO-MSLN-mAb}}\) was retained in the HT29-MSLN tumors from day 1 (10.67%ID/g) through day 3 (11.67%ID/g). Compared with days 1 and 3, tumor uptake of \(^{89}\text{Zr\text{-Zr-3,2-HOPO-MSLN-mAb}}\) declined to 7.97%ID/g.
FIG. 1. Structure of antibody conjugate and HPLC result. Structure of mesothelin antibody–chelator conjugate, 3,2-HOPO-MSLN-mAb (A) and DFO-MSLN-mAb (B). Representative HPLC of zirconium-89 labeled conjugates, \(^{89}\text{Zr}\)Zr-3,2-HOPO-MSLN-mAb (C), and \(^{89}\text{Zr}\)Zr-DFO-MSLN-mAb. (D) HPLC condition: eluent, 0.1 M sodium phosphate, 0.1 M sodium sulfate, 0.05% sodium azide, 10% isopropyl alcohol (pH 6.8), flow rate 0.3 mL/min; black line UV detector, red line radiodetector.

FIG. 2. In vitro saturation assay graphs. Representative in vitro saturation graphs of \(^{89}\text{Zr}\)Zr-3,2-HOPO-MSLN-mAb and \(^{89}\text{Zr}\)Zr-DFO-MSLN-mAb in HT29-MSLN, NCI-Meso16, and NCI-Meso21. For each plot Bt, bound total; Bnsp, bound nonspecific; Bsp, bound specific (Bt = Bnsp - Bsp).
on day 6 (Fig. 5A). Since radioactivity in the blood (%ID/g) decreased over the study period, Tissue:Blood ratios were calculated to normalize the difference in the input function. Tissue:Blood ratios (T:B; Fig. 5B) for tumor (day 1: 0.63; day 3: 1.22; day 6: 2.83) and femur (day 1: 0.37; day 3: 1.97; day 6: 5.06) increased over the study period with the highest ratios observed on day 6.

Since MSLN is not expressed in muscle, radioactivity associated with muscle was considered as background, thus Tissue:Muscle ratio was calculated to account for any interference due to background tissue radioactivity. Tissue:Muscle (T:M) ratios for tumor and femur increased over 6 d (Fig. 5C); however, this increasing T:M ratio is likely more indicative of the increased clearance of the radioactive conjugate from the muscle rather than an increased uptake in the tissue since the %ID/g in muscle decreased over the 6 d period. Approximately 80% (Fig. 5E) of the tumor uptake of \[^{89}\text{Zr}\]Zr-3,2-HOPO-MSLN-mAb could be blocked by the administration of excess of 3,2-HOPO-MSLN-mAb, indicating MSLN mediated accumulation of \[^{89}\text{Zr}\]Zr-3,2-HOPO-MSLN-mAb in the tumor.

**FIG. 3.** Mesothelin density per cell and binding affinity. Number of mesothelin per cell (A) and binding affinities (B; \(K_d\)) of \[^{89}\text{Zr}\]Zr-3,2-HOPO-MSLN-mAb and \[^{89}\text{Zr}\]Zr-DFO-MSLN-mAb for mesothelin were derived from the in vitro saturation assays in HT29-MSLN, NCI-Meso16, and NCI-Meso21 cells. The assay was performed on an average two to three times for each cell type.

| Cell Line | Mesothelin/Cell (\(10^3\)) |
|-----------|--------------------------|
| NCI-Meso16 | 178                      |
| NCI-Meso21 | 341                      |
| HT29-MSLN  | 249                      |

| Cell Line | Radiolabeled Antibody | \(K_d\) (nM) |
|-----------|-----------------------|--------------|
| NCI-Meso16 | \[^{89}\text{Zr}\]Zr-DFO-MSLN-mAb | 0.16±0.02 |
| NCI-Meso21 | \[^{89}\text{Zr}\]Zr-DFO-MSLN-mAb | 0.29±0.02 |
| HT29-MSLN  | \[^{89}\text{Zr}\]Zr-DFO-MSLN-mAb | 0.59±0.06 |
| NCI-Meso16 | \[^{89}\text{Zr}\]Zr-3,2-HOPO-MSLN-mAb | 2.1±0.09 |
| NCI-Meso21 | \[^{89}\text{Zr}\]Zr-3,2-HOPO-MSLN-mAb | 2.3±0.46 |
| HT29-MSLN  | \[^{89}\text{Zr}\]Zr-3,2-HOPO-MSLN-mAb | 1.9±0.39 |

**FIG. 4.** Competition binding assay and immunoreactivity plots. Representative plots from an in vitro \[^{89}\text{Zr}\]Zr-3,2-HOPO-MSLN-mAb (A) and \[^{89}\text{Zr}\]Zr-DFO-MSLN-mAb (B) competition-binding assays using unlabeled MSLN targeted monoclonal antibody (self-displacement, Morris method) with HT29-MSLN cells. Each point (average of duplicates) represents cell-bound CPM. Representative plots for determination of the % immunoreactivity (immunoreactive fraction) of \[^{89}\text{Zr}\]Zr-3,2-HOPO-MSLN-mAb (C) and \[^{89}\text{Zr}\]Zr-DFO-MSLN-mAb (D) from the same batch by the Morris method: representative plot (linear regression curve fit), immunoreactivity for \[^{89}\text{Zr}\]Zr-3,2-HOPO-MSLN-mAb=96% and \[^{89}\text{Zr}\]Zr-DFO-MSLN-mAb=95%. CPM, count per minute.
Biodistribution of the $[^{89}\text{Zr}]$Zr-3,2-HOPO-MSLN-mAb in HT29-MSLN xenograft was further compared with the biodistribution of $[^{89}\text{Zr}]$Zr-DFO-MSLN-mAb in the same tumor model (see Supplementary Table 2 for full biodistribution data). Over 6 d $[^{89}\text{Zr}]$Zr-DFO-MSLN-mAb either remained fairly constant (liver) or cleared rapidly from the nontarget tissue except for the femurs (Fig. 5A). Compared with day 1 (day 1: $3.57\%\text{ID/g}$), femur uptake of $[^{89}\text{Zr}]$Zr-DFO-MSLN-mAb increased at day 3 (day 3: $4.85\%\text{ID/g}$) and day 6 (day 6: $6.51\%\text{ID/g}$). However, when compared with $[^{89}\text{Zr}]$Zr-3,2-HOPO-MSLN-mAb, femur uptake of $[^{89}\text{Zr}]$Zr-DFO-MSLN-mAb was significantly ($2$- to $3.5$-fold, $p < 0.05$) lower over the study period (Fig. 5A). Tissue:Blood ratios of $[^{89}\text{Zr}]$Zr-3,2-HOPO-MSLN-mAb ($0.37$–$5.06$) and $[^{89}\text{Zr}]$Zr-DFO-MSLN-mAb ($0.15$–$1.14$) were higher compared with $[^{89}\text{Zr}]$Zr-3,2-HOPO-MSLN-mAb. The biodistribution patterns of both the conjugates in HT29-MSLN xenografts were further confirmed by PET/CT imaging results (Fig. 5D).

Biodistribution of the $[^{89}\text{Zr}]$Zr-3,2-HOPO-MSLN-mAb in HT29-MSLN xenograft was further compared with the biodistribution of $[^{89}\text{Zr}]$Zr-DFO-MSLN-mAb in the same tumor model (see Supplementary Table 2 for full biodistribution data). Over 6 d $[^{89}\text{Zr}]$Zr-DFO-MSLN-mAb either remained fairly constant (liver) or cleared rapidly from the nontarget tissue except for the femurs (Fig. 5A). Compared with day 1 (day 1: $3.57\%\text{ID/g}$), femur uptake of $[^{89}\text{Zr}]$Zr-DFO-MSLN-mAb increased at day 3 (day 3: $4.85\%\text{ID/g}$) and day 6 (day 6: $6.51\%\text{ID/g}$). However, when compared with $[^{89}\text{Zr}]$Zr-3,2-HOPO-MSLN-mAb, femur uptake of $[^{89}\text{Zr}]$Zr-DFO-MSLN-mAb was significantly ($2$- to $3.5$-fold, $p < 0.05$) lower over the study period (Fig. 5A). Moreover, femur T:B ratios (2.5- to 5-fold; Fig. 5B) and femur T:M ratios (2.3- to 5.5-fold; Fig. 5C) were higher for $[^{89}\text{Zr}]$Zr-3,2-HOPO-MSLN-mAb ($0.37$–$5.06$) than $[^{89}\text{Zr}]$Zr-DFO-MSLN-mAb ($0.15$–$1.14$) at all times.

Collectively, the data demonstrate decreased tumor accumulation of radioconjugate $3.2$-HOPO in vivo in comparison with radiolabeled DFO conjugate. Tumor uptake of $[^{89}\text{Zr}]$Zr-DFO-MSLN-mAb (day 1: $28.04\%\text{ID/g}$; day 3: $33.42\%\text{ID/g}$; day 6: $28.49\%\text{ID/g}$) was found to be $2.5$- to $3.5$-fold higher than tumor uptake of $[^{89}\text{Zr}]$Zr-3,2-HOPO-MSLN-mAb. Similarly, T:B (1.11–4.59) and T:M (14.46–40.23) tumor ratios of $[^{89}\text{Zr}]$Zr-DFO-MSLN-mAb were higher compared with $[^{89}\text{Zr}]$Zr-3,2-HOPO-MSLN-mAb. The biodistribution patterns of both the conjugates in HT29-MSLN xenografts were further confirmed by PET/CT imaging results (Fig. 5D).

Images obtained with $[^{89}\text{Zr}]$Zr-DFO-MSLN-mAb at days 1, 3, and 6 showed higher radioactivity in tumors compared with the images obtained with $[^{89}\text{Zr}]$Zr-3,2-HOPO-MSLN-mAb. Similar to the biodistribution studies, PET/CT images also displayed an increased uptake in the bone (joints and along the spinal cord; Fig. 5D) with $[^{89}\text{Zr}]$Zr-3,2-HOPO-MSLN-mAb vs $[^{89}\text{Zr}]$Zr-DFO-MSLN-mAb.

In a separate in vivo study, biodistribution of $[^{89}\text{Zr}]$Zr-3,2-HOPO-MSLN-mAb and $[^{89}\text{Zr}]$Zr-DFO-MSLN-mAb was performed in NCI-Meso16 and NCI-Meso21 xenografts, and compared with biodistribution of the conjugates in HT29-MSLN tumor models (Fig. 6A). Less radioactivity was measured in the blood of mice administered with $[^{89}\text{Zr}]$Zr-DFO-MSLN-mAb compared with $[^{89}\text{Zr}]$Zr-3,2-HOPO-MSLN-mAb (see Supplementary Table 3 for full biodistribution data).

For both conjugates, there was a difference in the amount of radioactivity measured in blood for different tumor types. This difference may result from the disparity in the amount of MSLN shed from each tumor in the blood. If the shed MSLN in the blood binds to the radioactive conjugate, it can interfere.
with the measure of the free radioactive conjugate in the blood.\textsuperscript{20} Compared with \[^{89}\text{Zr}]\text{Zr}-\text{DFO-MSLN-mAb}, \[^{89}\text{Zr}]\text{Zr}-3,2-\text{HOPO-MSLN-mAb} showed 3- to 6-fold higher radioactivity in the femur in all the xenograft models (Fig. 6A).

Throughout the study, tumor T:M ratios (Fig. 6B) were slightly higher (~1.3-fold) for \[^{89}\text{Zr}]\text{Zr}-\text{DFO-MSLN-mAb} compared with \[^{89}\text{Zr}]\text{Zr}-3,2-\text{HOPO-MSLN-mAb} in all tumor types. Contrary to tumor T:M, femur T:M ratios (Fig. 6B) of radioactive 3,2-HOPO conjugate were 6- to 10-fold higher than the radioactive DFO conjugate in all the three tumor models, suggesting the lower stability of the 3,2-HOPO chelate. Compared with \[^{89}\text{Zr}]\text{Zr}-3,2-\text{HOPO-MSLN-mAb}, \[^{89}\text{Zr}]\text{Zr-DFO-MSLN-mAb} consistently exhibited higher tumor uptake in all the three cancer models. Uptake (%ID/g) of \[^{89}\text{Zr}]\text{Zr-DFO-MSLN-mAb} in NCI-Meso16, NCI-Meso21, and HT29-MSLN tumors was 15.88, 19.49, and 33.41, respectively. Uptake of \[^{89}\text{Zr}]\text{Zr}-3,2-HOPO-MSLN-mAb in the three different tumors was found to be 7.95%ID/g (NCI-Meso16), 13.07%ID/g (NCI-Meso21), and 11.67%ID/g (HT29-MSLN).

Furthermore, imaging results in all three tumor xenografts (Fig. 6C) parallel the findings of day 3 biodistribution studies (Fig. 6A, B). The \[^{89}\text{Zr}]\text{Zr}-3,2-HOPO-MSLN-mAb PET/CT images demonstrated considerably higher uptake in bones (joints and spinal cord) than that observed in \[^{89}\text{Zr}]\text{Zr-DFO-MSLN-mAb} PET/CT images.

**Discussion**

In the present work, we synthesized and evaluated zirconium-89 labeled 3,2-HOPO-MSLN-mAb and DFO-MSLN-mAb both in vitro and in vivo. The radiochemical yield for \[^{89}\text{Zr}]\text{Zr}-3,2-HOPO-MSLN-mAb was slightly lower (80%–85%) than that for \[^{89}\text{Zr}]\text{Zr-DFO-MSLN-mAb} (90%–95%) under the same conditions. However, the amount of higher molecular weighted radiochemical aggregate was higher (30%) for \[^{89}\text{Zr}]\text{Zr}-3,2-HOPO-MSLN-mAb compared with \[^{89}\text{Zr}]\text{Zr-DFO-MSLN-mAb} (5%–18%). To obtain comparable radiochemical aggregates as \[^{89}\text{Zr}]\text{Zr-DFO-MSLN-mAb}, a lower concentration of 3,2-HOPO-MSLN-mAb per radiolabeling reaction was used. The lower radiochemical yield and purity of HOPO complexes have also been reported by Marik et al. for 3-hydroxypyridin-2-one (3,2-HOPO)-based dimacrocyclic ligand.\textsuperscript{22}

Both radiolabeled conjugates exhibited a high binding affinity for MSLN in all three cancer cell lines. Pharmacokinetics over the time course was similar for both the zirconium-89 labeled conjugates except for tumor and femur. As such, the in vivo biodistribution and PET/CT imaging studies demonstrated that \[^{89}\text{Zr}]\text{Zr-DFO-MSLN-mAb} exhibited 2.5- to 3.5-fold higher tumor uptake compared with \[^{89}\text{Zr}]\text{Zr-3,2-HOPO-MSLN-mAb} over 6 d.

Higher bone uptake with \[^{89}\text{Zr}]\text{Zr-3,2-HOPO-MSLN-mAb} conjugate is suggestive of the release of free zirconium-89(IV).\textsuperscript{38,39} In addition, the difference in the overall change and hydrophobicity of both the radioactive conjugates may contribute toward their diverse femur distribution.\textsuperscript{41} In vitro, human serum stability studies indicated that \[^{89}\text{Zr}]\text{Zr-3,2-HOPO-MSLN-mAb} was less stable compared with \[^{89}\text{Zr}]\text{Zr-DFO-MSLN-mAb}, with the caveat that the degradation products of the conjugates were not specifically identified.
It is important to mention that the biodistribution pattern of 3,2-HOPO-MSLN-mAb complexed to thorium-227 in HT29-MSLN tumor and femur was more similar to $[^{89}\text{Zr}]\text{Zr}-3,2$-HOPO-MSLN-mAb than $[^{89}\text{Zr}]\text{Zr}$-3,2-HOPO-MSLN-mAb.\textsuperscript{11} It is also necessary to acknowledge that 3,2-HOPO forms a stable complex with thorium-227, the $\alpha$-radionuclide used to develop $[^{227}\text{Th}]\text{Th}$-3,2-HOPO-MSLN-mAb therapeutic antibody conjugate.\textsuperscript{11,21}

Non-specific accumulation of radioactivity in the bone is highly undesirable.\textsuperscript{42} For instance, it can lead to (I) increased radiation dose to the bone marrow if this pattern is also seen in the thorium-227 antibody conjugate, and (II) possible interference with diagnosis and interpretation of cancer lesions metastasized to bones.\textsuperscript{42,43} Thus, it is important that the zirconium-89(IV) forms a stable complex with the chelate in vivo, and the conjugate exhibits biological stability in blood serum.

3,2-HOPO-based ligands belong to a class of octadentate chelators known as hydroxypyridinones, and various hydroxypyridinones or its modified version have been evaluated for their efficiency to chelate zirconium-89(IV).\textsuperscript{20,42,44–47} In a study, dimacrocyclic 3,2-HOPO chelate was conjugated to trastuzumab and compared with trastuzumab-DFO conjugate.\textsuperscript{22} In vitro serum stability indicated the percentage of intact 3,2-HOPO-based trastuzumab conjugate dropped to $\sim 50\%$ within the first 24 h. In vivo dimacrocyclic 3,2-HOPO labeled with zirconium-89 showed more radioactivity retained in the bone than DFO-based zirconium-89 complex.

The findings of this study align with the results of our studies. In addition to HOPO- and DFO-based chelators, other compounds such as hydroxyisophthalimide- and tetrazamacrocyclic-based ligands have been evaluated as zirconium-89 chelators to develop PET agents.\textsuperscript{38–50}

When plausible it is preferred that the same antibody conjugate is used to analyze the biodistribution in normal organs and lesion uptake as the one used to develop the therapeutic conjugate.\textsuperscript{51} Therefore, this study intended to compare the biodistribution of $[^{89}\text{Zr}]\text{Zr}$-3,2-HOPO-MSLN-mAb and $[^{89}\text{Zr}]\text{Zr}$-DFO-MSLN-mAb. Findings of this study comparing $[^{89}\text{Zr}]\text{Zr}$-3,2-HOPO-MSLN-mAb and $[^{89}\text{Zr}]\text{Zr}$-DFO-MSLN-mAb suggest that even though $[^{89}\text{Zr}]\text{Zr}$-3,2-HOPO-MSLN-mAb can accumulate in tumors expressing high levels of MSLN, its utility in detecting low-density MSLN tumor lesions might be limited. Moreover, the higher bone uptake of $[^{89}\text{Zr}]\text{Zr}$-3,2-HOPO-MSLN-mAb may interfere with the identification of any bone metastasis.

**Conclusion**

$[^{89}\text{Zr}]\text{Zr}$-DFO-MSLN-mAb showed a higher tumor and lower femur uptake than $[^{89}\text{Zr}]\text{Zr}$-3,2-HOPO-MSLN-mAb. However, because $[^{89}\text{Zr}]\text{Zr}$-3,2-HOPO-MSLN-mAb uses the same chelator as $[^{227}\text{Th}]\text{Th}$-3,2-HOPO-MSLN-mAb, the same zirconium-89 labeled 3,2-HOPO-MSLN-mAb conjugate could be better at studying organ distribution and lesion uptake of the MSLN-TTC, with the caveat that detection of MSLN-positive tumors in the lower extremity might be more difficult if high femur uptake is also seen in humans.

**Authorship Confirmation Statement**

J.R. contributed to study conception and design, conducting experiment and acquiring data, analyzing and interpreting data, drafting the article, and critical revision; E.M.J. contributed to study conception and design, conducting experiment and acquiring data, reviewing the article, and critical revision; F.B. and O.V. contributed to synthesis and radiolabeling of antibody zirconium-89 conjugates, and critical revision; T.E.P., K.W., A.T.T., and P.L.C. contributed to conducting experiment and acquiring data, and critical revision; U.B.H. contributed to study conception and design, review data, and critical revision; A.S.C. and P.E.C. contributed to review data and critical revision; R.H. contributed to study conception and design, and critical revision; F.I.L. contributed to study conception and design, review data, drafting the article, and critical revision.

As a corresponding author of this article (F.I.L.), I confirm that all the coauthors have approved this article as submitted.

**Disclosure Statement**

A.S.C. holds a patent on 3,2-HOPO-MSLN-mAb conjugate. Other authors declare that he/she has no potential conflict of interest.

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**Supplementary Material**

Supplementary Data

Supplementary Table S1

Supplementary Table S2

Supplementary Table S3

Supplementary Figure S1

Supplementary Figure S2

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