
Diabody Pretargeting with Click Chemistry In Vivo

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Radioimmunotherapy and nuclear imaging (immuno-PET/SPECT) of cancer with radiometal-labeled antibody fragments or peptides is hampered by low tumor-to-kidney ratios because of high renal radiometal retention. Therefore, we developed and evaluated a pretargeting strategy using click chemistry in vivo to reduce kidney uptake and avoid unwanted radiation toxicity. We focused on the bioorthogonal reaction between a *trans*-cyclooctene (TCO)-functionalized TAG72 targeting diabody, AVP04-07, and a low-molecular-weight radiolabeled tetrazine probe that was previously shown to have low kidney retention and relatively fast renal clearance. **Methods:** AVP04-07 diabodies were functionalized with TCO tags, and in vitro immunoreactivity toward bovine submaxillary mucin and tetrazine reactivity were assessed. Next, pretargeting biodistribution studies were performed in LS174T tumor-bearing mice with AVP04-07-TCO(*n*) (where *n* indicates the number of TCO groups per diabody) and radiolabeled tetrazine to optimize the TCO modification grade (0, 1.8, or 4.7 TCO groups per diabody) and the ¹⁷⁷Lu-tetrazine dose (0.1, 1.0, or 10 Eq with respect to the diabody). Radiolabeled tetrazine was injected at 47 h after diabody injection, and mice were euthanized 3 h later. A pretargeting SPECT/CT study with ¹¹¹In-tetrazine was performed with the optimized conditions. **Results:** Immunoreactivity for native AVP04-07 was similar to that for TCO-functionalized AVP04-07, and the latter reacted efficiently with radiolabeled tetrazine in vitro. The combination of the pretargeting component AVP04-07 functionalized with 4.7 TCO groups and 1 Eq of ¹⁷⁷Lu-tetrazine with respect to the diabody showed the most promising biodistribution. Specifically, high ¹⁷⁷Lu-tetrazine tumor uptake (6.9 percentage injected dose/g) was observed with low renal retention, yielding a tumor-to-kidney ratio of 5.7. SPECT/CT imaging confirmed the predominant accumulation of radiolabeled tetrazine in the tumor and low nontumor retention. **Conclusion:** Pretargeting provides an alternative radioimmunotherapy and nuclear imaging strategy by overcoming the high renal retention of low-molecular-weight radiometal tumor-homing agents through the separate administration of a tumor-homing agent and a radioactive probe with fast clearance.

Key Words: Diels–Alder; click; pretargeting; diabody

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Target-specific radiolabeled molecules that show rapid tumor targeting and fast clearance from blood and normal tissues are of great interest for nuclear imaging and radiotherapy of solid tumors. Intact monoclonal antibodies (mAbs) generally exhibit superb tumor uptake and retention, but these properties are offset by the long residence time in blood, which results in low tumor-to-blood ratios and radiation dose-limiting side effects in bone marrow (1). Alternatively, low-molecular-weight proteins (with masses of <60 kDa) and peptides such as Affibody molecules, Nanobodies, single-chain Fv fragments, and noncovalent stable dimers of single-chain Fv fragments (diabodies) are rapidly cleared from the blood by the kidneys and can provide better tumor-to-nontumor ratios at shorter time intervals than mAbs, albeit usually in combination with lower target uptake (2). However, on glomerular filtration, peptides and antibody fragments are generally reabsorbed by the proximal tubule cells in the kidneys to prevent the loss of valuable amino acids, and this step is followed by lysosomal degradation (3). With regard to radiometal-labeled agents, the catabolic radiometal products have been shown to have prolonged renal entrapment, which typically hampers nuclear imaging and radioimmunotherapy procedures with these agents because of possible nephrotoxicity (4–10). Therefore, strategies that reduce the renal uptake of radiometal-labeled low-molecular-weight agents to enable the administration of higher radiation doses with reduced risk for nephrotoxicity remain of great interest.

In recent years, several strategies that reduce the renal uptake of radiometal-labeled low-molecular-weight agents have been developed (6,11–21). One example is the conjugation of polyethylene glycol (PEG) to antibody fragments to increase the molecular weight beyond the renal molecular weight cutoff of 60 kDa, resulting in an impressive reduction in kidney uptake (21,22). However, because of the reduction in glomerular filtration, these PEGylated antibody fragments have a relatively long circulation time. Therefore, there is still a need for a way to use peptides or small proteins for radioimaging or therapy without having to resort to cumbersome and case-by-case optimization of the structure and while still retaining the fast clearance of small molecules. Here, we focus on the use of an in vivo pretargeting strategy with minimal perturbation of the parent structure and pharmacokinetics (23).

Pretargeting strategies involve the separate administration of a tumor-binding agent, which binds to a tumor-specific extracellular antigen, and a radionuclide. In the first step, the tumor-binding agent is administered and accumulates in the tumor. On blood clearance of the circulating tumor-binding agent, a radiolabeled probe with fast clearance is administered. The probe efficiently targets the radioactivity to the prelocalized agent in the tumor, and unreacted probe is

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rapidly excreted from the circulation, with low renal retention. We previously showed that antibody pretargeting through *in vivo* click chemistry results in higher tumor-to-nontumor ratios than conventional radiolabeled mAbs (24). In that study, we used a tumor pretargeting strategy based on the bioorthogonal inverse-electron-demand Diels–Alder reaction between a highly reactive *trans*-cyclooctene (TCO)-tagged TAG72 binding antibody and radiometal-labeled tetrazine. Because of the long circulation time of antibodies, clearing agents were injected to effectively remove residual antibodies from the blood before radiolabeled tetrazine was administered. Radiolabeled tetrazine displayed high tumor uptake, low kidney retention, and relatively fast excretion from the kidneys over time (24).

We hypothesized that translation of the pretargeting strategy to smaller proteins and peptides might be an attractive approach to overcoming the high kidney radiation dose associated with the renal reabsorption of tumor-homing agents labeled with a radiometal. In comparison with our previous pretargeting work on antibodies with a long circulation time (23–25), the relatively fast clearance of low-molecular-weight agents is expected to result in high tumor-to-blood ratios without the need for clearing agents. Among antibody fragments, diabodies (~55 kDa) are of particular interest because they provide greater avidity than other agents, including monovalent single-chain Fv fragments, and therefore typically show higher tumor uptake (2,21,22). In the present study, we explored the use of a TAG72-binding diabody, AVP04-07 (21), as a model construct for a pretargeting approach in LS174T tumor-bearing mice (Fig. 1A). The results of the diabody pretargeting approach were compared with previously published data for a conventional radiometal-labeled TAG72-binding diabody analog that showed high tumor targeting and fast blood clearance but high renal retention (21).

MATERIALS AND METHODS

All reagents and solvents were obtained from commercial sources (Sigma-Aldrich, Acros, Invitrogen, and Merck) and used without further purification unless stated otherwise. ¹¹¹In-indium chloride, ¹⁷⁷Lu-lutetium chloride, and sodium ¹²⁵I-iodide solutions were purchased from PerkinElmer. Water was distilled and deionized (18 MΩcm) by

means of a milli-Q water filtration system (Millipore). The labeling buffers were treated with Chelex-100 resin (BioRad Laboratories) overnight, filtered through 0.22-μm filters, and stored at 4°C. Bolton–Hunter reagent (*N*-succinimidyl-3-[4-hydroxyphenyl]propionate [SHPP]), Gelcode Blue protein staining solutions, and Zeba desalting spin columns (7-kDa cutoff; 0.5 mL) were purchased from Pierce Protein Research (Thermo Fisher Scientific). Mouse serum was purchased from Innovative Research.

The ¹¹¹In and ¹⁷⁷Lu labeling yields for DOTA–tetrazine were determined by radio–thin-layer chromatography (radio-TLC) with ITLC-SG strips (Varian Inc.) eluted with 200 mM ethylenediaminetetraacetic acid in saline and imaged on a phosphorimager (FLA-7000; Fujifilm). Under these conditions, free ¹¹¹In and ¹⁷⁷Lu migrated with an R_f value of 0.9, whereas ¹¹¹In- or ¹⁷⁷Lu-tetrazine remained at the origin. The radiochemical purity of ¹¹¹In- and ¹⁷⁷Lu-labeled DOTA–tetrazine was determined by radio–high-pressure liquid chromatography (HPLC) on an Agilent 1100 system equipped with a Gabi radioactive detector (Raytest). The samples were loaded on an Agilent Eclipse XDB-C18 column (4.6 × 150 mm, 5 μm) and eluted at 1 mL/min with a linear gradient of acetonitrile in water containing 0.1% trifluoroacetic acid (2 min at 10% acetonitrile followed by an increase to 45% acetonitrile in 11 min). The ultraviolet wavelength was preset at 254 nm. The ¹²⁵I-diabody labeling yields were determined by radio-TLC with ITLC-SG strips eluted with a 1:1 methanol–ethyl acetate mixture and imaged on a phosphorimager. Under these conditions, free ¹²⁵I-iodide and ¹²⁵I-SHPP migrated with R_f values of 0.5–0.9, whereas ¹²⁵I-diabodies remained at the origin. The radiochemical purity of ¹²⁵I-AVP04-07 and TCO-conjugated ¹²⁵I-AVP04-07 was determined by size exclusion chromatography (SEC) and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). SEC was performed on an Agilent 1200 system equipped with a Gabi radioactive detector (Raytest). The samples were loaded on a Superdex 200 10/300 column (GE Healthcare) and eluted with 10 mM phosphate buffer (pH 7.4) at 0.5 mL/min. The ultraviolet wavelength was preset at 260 and 280 nm. SDS-PAGE was performed on a Phastgel system with 4%–15% PAGE gradient gels (GE Healthcare).

Syntheses of Pretargeting Components

The synthesis of DOTA–tetrazine (Fig. 1B) (24), the synthesis of the axial isomer of TCO–oxymethylbenzamide-*N*-hydroxysuccinimide (26), and AVP04-07 production have been described elsewhere. AVP04-07, a stable, approximately 55-kDa diabody format of monoclonal antibody CC49, was produced by dimerization of single-chain Fv fragments with short (5-residue) linkers in a bacterial periplasm fermentation system (21,26).

Synthesis of AVP04-07–TCO(n)

AVP04-07 (1 mg; a solution of 6 mg/mL in phosphate-buffered saline [PBS]) was modified with 2 or 5 M Eq of the axial isomer of TCO–oxymethylbenzamide-NHS (10 mg/mL in dimethyl sulfoxide) in a total volume of 250 μL of PBS. The pH was adjusted to 9 with 1 M sodium carbonate buffer. The reactions were performed under agitation for 30 min at room temperature in the dark. Subsequently, the obtained AVP04-07–TCO(n) (where *n* indicates the number of TCO groups per diabody) was purified twice through Zeba desalting spin columns (preequilibrated with PBS), and the concentration of the obtained solution was measured with NanoDrop (Thermo Scientific). TCO loading was revealed from the reaction yield between AVP04-07–TCO(n) (10 μg in 50 μL of PBS) and 3 or 7 Eq of radiolabeled tetrazine (20 min of incubation at 37°C and 300 rpm).

DOTA–Tetrazine Radiolabeling

DOTA–tetrazine was dissolved (1.5 mg/mL; 1.17 mM) in 0.2 M ammonium acetate buffer (pH 7.0) and stored at –80°C before use. An aliquot of DOTA–tetrazine was mixed with a suitable amount of ¹⁷⁷LuCl₃ or ¹¹¹InCl₃ in 0.2 M ammonium acetate (pH 5.5) and incubated

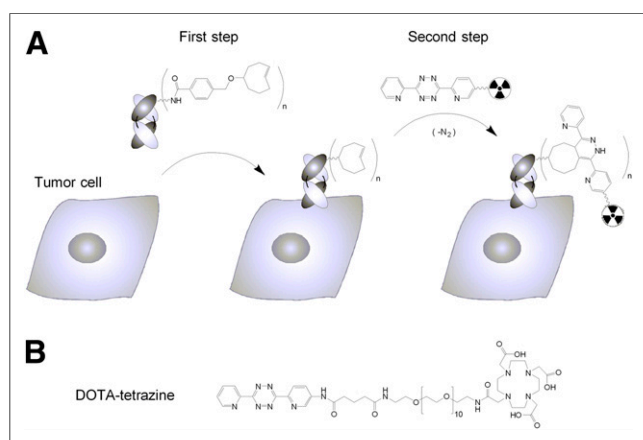


FIGURE 1. (A) Schematic of diabody tumor pretargeting strategy based on inverse-electron-demand Diels–Alder reaction between TCO-tagged diabody and radiolabeled tetrazine. On tumor binding and blood clearance of diabody, administration of radiolabeled tetrazine targets radioactivity to prelocalized diabody in tumor. (B) Molecular structure of DOTA–tetrazine.

at 60°C for 5 min. Next, the labeling mixture was mixed with a 10 mM diethylenetriaminepentaacetic acid solution (5 μ L) and a gentisic acid solution (20 mg/mL) in saline at pH 6.5 and incubated for 5 min. The radiochemical yield and radiochemical purity were assessed by radio-TLC and radio-HPLC, respectively, after which the labeling mixture was diluted with sterile saline for animal experiments. The specific activity of ^{177}Lu -tetrazine used for biodistribution and that of ^{111}In -tetrazine used for SPECT/CT imaging were 0.07–7 MBq/nmol and approximately 60 MBq/nmol, respectively.

AVP04-07-TCO(n) Radiolabeling

Radioiodination of AVP04-07-TCO(n) diabodies was performed with the Bolton–Hunter method in accordance with manufacturer instructions. In brief, a suitable amount of sodium ^{125}I -iodide in 50 μ L of PBS was mixed with 0.1 μ g of Bolton–Hunter reagent (SHPP) and 100 μ g of chloramine-T (*N*-chloro-4-methylbenzenesulfonamide, sodium salt). The resulting solution was mixed for 10–20 s, after which ^{125}I -SHPP was extracted in toluene and the organic solution was blown to dryness under a gentle stream of N_2 . AVP04-07-TCO(n) (0.1 mg in 50 μ L of PBS) was added to the dry ^{125}I -SHPP, the pH was adjusted to 9 with 1 M sodium carbonate buffer, and the reaction mixture was incubated at room temperature for 30–60 min with gentle shaking. After incubation, the labeling yields were determined by radio-TLC. The ^{125}I -labeled diabodies were purified twice through Zeba spin desalting columns (preequilibrated with saline) in accordance with manufacturer instructions, and the radiochemical purity was determined by radio-TLC, SEC, and SDS-PAGE. For animal experiments, the specific activity of ^{125}I -AVP04-07-TCO(n) was adjusted to 12–15 kBq/ μ g by the addition of cold diabody and sterile saline.

Immunoreactivity

^{125}I -labeled AVP04-07 and AVP04-07-TCO(n) were reacted with 20 Eq of bovine submaxillary mucin (BSM, a surrogate target antigen for human TAG72) in 1% bovine serum albumin in PBS at 37°C for 20 min at 350 rpm. Next, the reaction mixtures were analyzed by SEC to identify and quantify diabodies and diabody–BSM complexes.

Animal Experiments

All animal experiments were performed in accordance with the principles of laboratory animal care (NIH publication 85-23, revised 1985) and Dutch national law Wet op de Dierproeven (Stb 1985, 336). The *in vivo* experiments were performed in tumor-bearing nude female BALB/c mice (body weight, 20–25 g; Charles River Laboratories). Human colon cancer cell line LS174T was obtained from the American Type Culture Collection and maintained in Eagle minimal essential medium (Sigma) supplemented with 10% heat-inactivated fetal calf serum (Gibco), penicillin (100 U/mL), streptomycin (100 μ g/mL), and 2 mM GlutaMAX (Gibco). Mice were inoculated subcutaneously with 5×10^6 cells in 100 μ L of sterile PBS and were used 7–10 d after tumor inoculation, when the tumors reached a size of approximately 70–200 mm³. At the end of each experiment, the mice were anesthetized and euthanized by cervical dislocation. Blood was withdrawn by heart puncture, and selected organs and tissues were harvested and blotted dry. All samples were weighed and then combined with 1 mL of PBS. The sample radioactivity was counted in a γ counter (Wizard 1480; PerkinElmer) along with standards to determine the percentage injected dose/g (%ID/g) and the percentage injected dose per organ. The tissues from dual-isotope experiments were measured by use of a dual-isotope protocol (energy windows of 10–80 and 155–380 keV for ^{125}I and ^{177}Lu , respectively) with cross-contamination correction.

Blood Clearance and Biodistribution Experiments

Three groups of 3 LS174T tumor-bearing mice were injected intravenously with ^{125}I -AVP04-07, ^{125}I -AVP04-07-TCO(1.8), and ^{125}I -AVP04-07-TCO(4.7) (35 μ g/100 μ L/mouse; \sim 0.2 MBq). The

mice were serially bled at 5 min, 30 min, 1 h, 2 h, 6 h, and 24 h. Next, the mice were injected with 6.7 nmol of ^{177}Lu -tetrazine (8.52 μ g/80 μ L/mouse; containing 100 μ g of gentisic acid; \sim 0.5 MBq) at 47 h after diabody injection and euthanized at 3 h after tetrazine injection, and organs and tissues of interest were harvested. Two groups of 4 other LS174T tumor-bearing mice were injected intravenously with ^{125}I -AVP04-07-TCO(4.7) (35 μ g/100 μ L/mouse; \sim 0.2 MBq), and 0.67 nmol or 67 pmol of ^{177}Lu -tetrazine was administered at 47 h after diabody injection. The mice were euthanized at 3 h after tetrazine injection, and organs and tissues of interest were harvested. The radioactivity in blood samples, organs, and tissues was counted. Blood clearance data were fitted with a 2-phase exponential decay function, and the area under the curve was determined. Blood half-lives subsequently were derived with the formula $\ln 2 \times \text{AUC}/C_0$, where AUC is the area under the curve and C_0 represents the probe concentration (%ID/g) in blood at time 0.

SPECT/CT Imaging Experiment

One LS174T tumor-bearing mouse was injected with AVP04-07-TCO(4.7) (35 μ g/100 μ L) and ^{111}In -tetrazine (0.67 nmol/80 μ L; containing 100 μ g of gentisic acid; \sim 42 MBq) after the pretargeting protocol. At approximately 90 min after tetrazine injection, the mouse was anesthetized with isoflurane and imaged on a dedicated small-animal SPECT/CT system (NanoSPECT/CT; Bioscan) equipped with 4 detector heads and converging 9-pin-hole collimators (pin-hole diameter, 1.4 mm). First, a CT scan was performed (180 projections; 1,000 ms/projection; 45-kV peak tube voltage; 177-mA tube current; 35-mm field of view), and then a SPECT scan was performed (24 projections; 120 s/projection; photopeaks for ^{111}In set to 171 keV [15% full width] and 245 keV [20% full width]). At 3 h after tetrazine injection, the mouse was euthanized by anesthesia overdose, and a high-resolution SPECT/CT scan was performed (360 projections and 2,000 ms/projection for CT; 36 projections and 300 s/projection for SPECT). The SPECT image was reconstructed with HiSPECTNG (SciVis GmbH) to an isotropic voxel size of 300 μ m. The CT image was reconstructed with InVivoScope (Bioscan) to an isotropic voxel size of 200 μ m.

Data Analysis

The data for %ID/g or percentage injected dose per organ are presented as mean \pm 1 SD. Standard 1-way ANOVAs with Bonferroni post hoc testing for multiple group comparisons, curve fitting, and calculation of the area under the curve were performed with GraphPad Prism version 4.1. The difference between 2 data points was considered statistically significant when the *P* value was less than 0.05.

RESULTS

Diabody Functionalization, Radiochemistry, and In Vitro Characterization

The TAG72-targeting diabody AVP04-07 was reacted with TCO-oxymethylbenzamide-NHS (26) through lysine residue conjugation (Supplemental Fig. 1) (supplemental materials are available at <http://jnm.snmjournals.org>) with the aim of affording 2 distinct modification ratios within the range of approximately 2–5 TCO groups per diabody. Radioiodination of AVP04-07 and AVP04-07-TCO was performed with the Bolton–Hunter reagent in PBS. The procedure produced a 65%–70% labeling yield and, after size exclusion purification, the radiolabeled diabodies had a radiochemical purity of greater than 98%, as confirmed by radio-TLC and SDS-PAGE analysis (Supplemental Figs. 2 and 3). DOTA-tetrazine was labeled with ^{177}Lu for *in vitro* reactivity assays and biodistribution experiments or ^{111}In for SPECT/CT imaging, with a labeling yield greater than 99% and a radiochemical purity greater than 95%

(Supplemental Figs. 3 and 4). The reaction yield for AVP04-07-TCO(*n*) and 3 or 7 Eq of radiolabeled tetrazine indicated the presence of, on average, 1.8 or 4.7 TCO groups per diabody, respectively. In an immunoreactivity assay, iodinated AVP04-07-TCO showed quantitative binding to 20 Eq of TAG72-positive BSM (Supplemental Fig. 5).

Optimization of Diabody TCO Loading for In Vivo Pretargeting Experiments

To determine the optimal diabody TCO loading, we injected 3 ¹²⁵I-labeled AVP04-07 constructs, containing 0, 1.8, and 4.7 TCO groups per diabody, into LS174T tumor-bearing mice. Blood clearance data showed 1.7- and 1.9-fold-slower blood clearance for ¹²⁵I-AVP04-07-TCO(1.8) and ¹²⁵I-AVP04-07-TCO(4.7), respectively, than for native ¹²⁵I-AVP04-07 (Fig. 2). All diabodies were essentially cleared from the circulation at 50 h after diabody injection. At 47 h after diabody injection, 10 Eq of ¹⁷⁷Lu-tetrazine (with respect to the diabody) were administered by intravenous injection. The dual-isotope biodistribution results at 3 h after ¹⁷⁷Lu-tetrazine injection, corresponding to 50 h after diabody injection, were similar for the 3 diabodies (Fig. 3A; Supplemental Table 1). Specifically, the ¹²⁵I-diabodies showed high tumor uptake (12–14 %ID/g), whereas normal tissues and organs showed low to modest levels (~0.05 %ID/g for muscle, ~0.8 %ID/g for spleen, and 2.2–2.9 %ID/g for liver). The low ¹²⁵I levels in the stomach and thyroid indicated no significant in vivo dehalogenation of the ¹²⁵I-AVP04-07 constructs.

The administration of 10 Eq of ¹⁷⁷Lu-tetrazine led to low tumor uptake for mice pretargeted with native AVP04-07, whereas with AVP04-07-TCO(1.8) and AVP04-07-TCO(4.7), the ¹⁷⁷Lu-tetrazine tumor levels were significantly higher and correlated with the TCO concentration (0.78 ± 0.20 and 1.55 ± 0.22 %ID/g, respectively) (Supplemental Table 1). Calculation of the on-tumor reaction yields (based on TCO) for the ¹²⁵I-labeled diabody-TCO constructs and ¹⁷⁷Lu-tetrazine revealed values of 31.2% ± 7.2% and 37.7% ± 3.1% for AVP04-07-TCO(1.8) and AVP04-07-TCO(4.7), respectively. For all other tissues and organs, the ¹⁷⁷Lu-tetrazine retention was low to very low and independent of the preadministered diabody. Because the tumor-to-nontumor ratios for ¹⁷⁷Lu-tetrazine in mice pretargeted with AVP04-07-TCO(4.7) were significantly

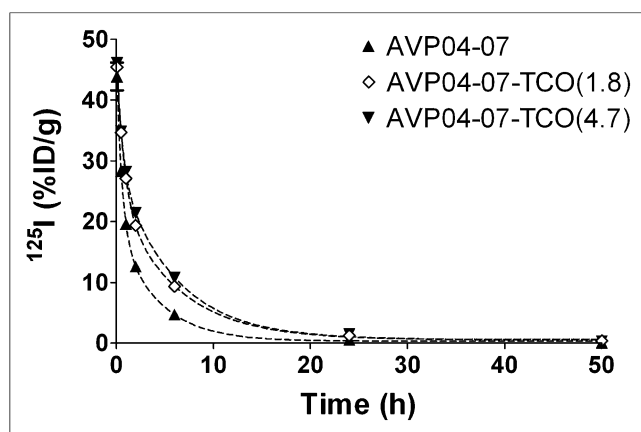


FIGURE 2. Blood clearance of 35 µg of ¹²⁵I-AVP04-07, ¹²⁵I-AVP04-07-TCO(1.8), and ¹²⁵I-AVP04-07-TCO(4.7) in LS174T tumor-bearing mice. Data points represent mean ± SD %ID/g (*n* = 3). Half-lives were 1.92 h for ¹²⁵I-AVP04-07, 3.30 h for ¹²⁵I-AVP04-07-TCO(1.8), and 3.58 h for ¹²⁵I-AVP04-07-TCO(4.7).

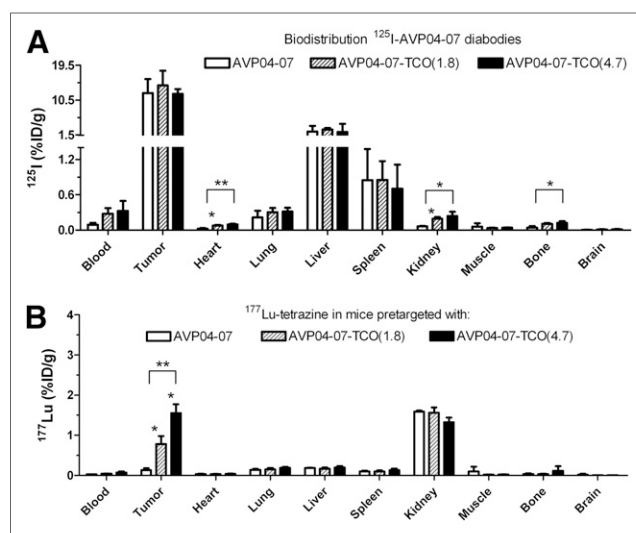


FIGURE 3. Dual-isotope biodistribution for pretargeting with ¹²⁵I-AVP04-07, ¹²⁵I-AVP04-07-TCO(1.8), or ¹²⁵I-AVP04-07-TCO(4.7) and ¹⁷⁷Lu-tetrazine in LS174T tumor-bearing mice. Mice were injected with one of the ¹²⁵I-AVP04-07 diabodies, and ¹⁷⁷Lu-tetrazine (10 Eq, with respect to diabody) was administered at 47 h after diabody injection. Mice were euthanized 3 h later. (A) Biodistribution of ¹²⁵I-AVP04-07 diabodies. (B) Biodistribution of ¹⁷⁷Lu-tetrazine. Bars represent mean ± SD %ID/g (*n* = 3). **P* < 0.05. ***P* < 0.001.

higher for most tissues than those obtained with AVP04-07-TCO(1.8) (Supplemental Table 2), the former was selected for further evaluation.

Optimization of ¹⁷⁷Lu-Tetrazine Dose to Improve Tumor Uptake and SPECT/CT Imaging

To investigate whether tumor uptake and the tumor-to-blood or tumor-to-normal tissue ratios could be further improved, we investigated 2 additional dose levels. Pretargeting biodistribution studies with 1.0 and 0.1 Eq of ¹⁷⁷Lu-tetrazine (with respect to the diabody) administered at 47 h after diabody injection were performed in mice pretargeted with ¹²⁵I-AVP04-07-TCO(4.7) (Fig. 4A; Supplemental Table 3). Although the tumor-to-blood ratio decreased with decreasing amounts of injected ¹⁷⁷Lu-tetrazine (Fig. 4B), most other tumor-to-organ ratios were significantly higher when 1 Eq of ¹⁷⁷Lu-tetrazine was administered with respect to AVP04-07-TCO(4.7) than when either 0.1 or 10 Eq were administered (Supplemental Table 4). Specifically, the tumor-to-kidney ratio increased from 1.2 to 5.7 when 1 instead of 10 Eq of ¹⁷⁷Lu-tetrazine was administered. In addition, tumor uptake was maximal at that dose (6.9 ± 1.1 %ID/g). Therefore, 1 Eq of ¹⁷⁷Lu-tetrazine (with respect to the diabody) was considered to be the optimal ¹⁷⁷Lu-tetrazine dose.

A subsequent SPECT/CT imaging experiment with the pretargeting protocol at this optimal dose confirmed high tumor uptake of ¹¹¹In-tetrazine and low retention in nontarget organs (Fig. 5). This finding was expected because we and others previously demonstrated similar biodistributions of ¹⁷⁷Lu- and ¹¹¹In-labeled mAbs and low-molecular-weight agents when DOTA was used as a chelator (25,27–30).

DISCUSSION

The tubular reabsorption and subsequent high renal retention of radiometal-labeled peptides, antibody fragments, and other small proteins hamper their use in nuclear imaging and targeted radiotherapy of cancer because of possible nephrotoxicity. Therefore,

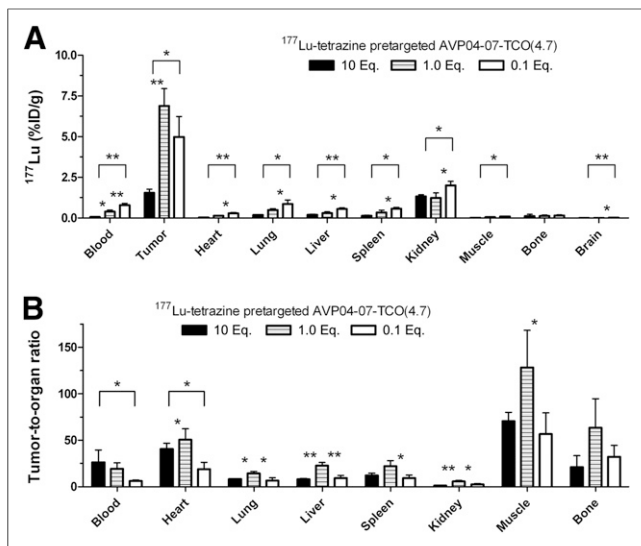


FIGURE 4. ^{177}Lu -tetrazine biodistribution in LS174T tumor-bearing mice pretargeted with AVP04-07-TCO(4.7). Mice were injected with AVP04-07-TCO(4.7), and ^{177}Lu -tetrazine (10 Eq [$n = 3$], 1.0 Eq, or 0.1 Eq [$n = 4$], with respect to diabody) was administered at 47 h after diabody injection. Mice were euthanized 3 h later. (A) Biodistribution of ^{177}Lu -tetrazine. (B) Tumor-to-organ ratios for ^{177}Lu -tetrazine. Bars represent mean \pm SD. * $P < 0.05$. ** $P < 0.001$.

various strategies that aim to reduce renal uptake of radiometal-labeled low-molecular-weight cancer-targeting agents have been developed. Coadministration of cationic amino acids has been shown to inhibit tubular reabsorption of radiolabeled peptides and antibody fragments by up to 35%–40% (6,11,12). Coinfusion of the plasma expander Gelofusine (with or without coadministration of cationic amino acids) also resulted in a strong reduction in kidney uptake for a radiolabeled octreotate (31) and a radiolabeled Nanobody (28,29). In another approach, radiolabeled chelates were designed to be cleaved from the protein or peptide on glomerular filtration but before reabsorption, allowing renal excretion of the radiolabel. This strategy resulted in a 75% reduction in renal uptake of the radiolabeled chelate (14). Importantly, none of these approaches could completely inhibit the renal reabsorption of small radiolabeled agents and, for the approaches that have been evaluated in tumor-bearing animals, kidney retention was still higher than tumor uptake (14,32,33). Therefore, more efficient strategies for reducing radiation toxicity in the kidneys are needed.

Interestingly, approaches focused on increasing the apparent size of the targeting agents did reduce the glomerular filtration rate and thereby decreased renal reabsorption and strongly improved radionuclide tumor-to-kidney ratios (15,21). For example, both the PEGylation of diabodies and the modification of Affibody molecules with albumin-binding domains resulted in a strong reduction in glomerular filtration and tumor uptake that was 7-fold higher than kidney uptake (in %ID/g) (15,21). However, these strategies are hampered by the prolonged residence time of the agents in blood, resulting in relatively low tumor-to-blood ratios and the risk of radiation toxicity for bone marrow, similar to that observed with intact antibodies.

We previously developed a click chemistry pretargeting strategy that may be an attractive alternative to reengineering (24). An anti-TAG72 diabody, AVP04-07, was selected as a low-molecular-weight tumor-homing model construct. Previous work showed that



FIGURE 5. SPECT/CT image (maximum-intensity projection) of ^{111}In -tetrazine in LS174T tumor-bearing mouse pretargeted with AVP04-07-TCO(4.7). Image shows high radioactivity uptake in tumor (hind limb) and bladder (urine). Low retention of radioactivity was observed in kidneys, whereas all other organs and tissues showed negligible levels of radioactivity.

body. In addition, the diabody-TCO constructs reacted efficiently with the ^{177}Lu -radiolabeled tetrazine probe in PBS. In mice, the different radioiodinated diabodies [^{125}I -AVP04-07-TCO(n)] had comparable biodistribution, indicating that the modification of the diabody with a TCO tag did not significantly perturb its pharmacokinetics.

We observed a relatively minor increase in the blood clearance half-life for the TCO-modified diabodies compared with the native diabody; this increase was most likely due to noncovalent or covalent albumin binding (34,35). The similar tumor uptake for native and tagged diabodies might be explained by a slightly reduced tumor bioavailability for the diabody fraction that is bound to albumin, counteracting the slightly longer blood circulation time and expected increased tumor exposure and binding. After the administration of ^{177}Lu -radiolabeled tetrazine and dual-isotope biodistribution analysis, we found on-tumor reaction yields (based

although a radiolabeled DOTA conjugate of this diabody efficiently targeted the tumor in LS174T tumor-bearing mice, renal retention of the radiometal label was high (21), resulting in a problematic tumor-to-kidney ratio of 0.25 at 48 h after injection. PEGylation of the AVP04-07 diabody diminished kidney uptake and increased blood circulation, boosting tumor uptake from 21 to 44 %ID/g and yielding a much improved tumor-to-kidney ratio of 7. However, as a result of the longer circulation half-life, the tumor-to-blood ratio at 48 h after injection was 4.4, whereas that for native AVP04-07 was greater than 50.

We set out to develop a strategy that affords a practical tumor-to-kidney ratio of greater than 4 while maintaining the high tumor-to-blood ratios typical of low-molecular-weight agents such as diabodies. We applied our previously developed click chemistry pretargeting approach to the AVP04-07 diabody by using TCO-modified AVP04-07 diabody and radiolabeled tetrazine. The diabody constructs, comprising 1.8 and 4.7 TCO groups per diabody, retained their immunoreactivity toward BSM (compared with the non-modified diabody); this finding demonstrated that the small TCO tag—when bound to surface lysine residues—was well tolerated by the dia-

on TCO) of $31.2\% \pm 7.2\%$ and $37.7\% \pm 3.1\%$ for AVP04-07–TCO(1.8) and AVP04-07–TCO(4.7), respectively; these values correspond well to the value of 46% found in an earlier pretargeting study with the same molar dose of full mAb CC49 modified with 8 TCO groups (24). Because ^{177}Lu -tetrazine showed the highest tumor accumulation in animals pretargeted with AVP04-07–TCO(4.7) and the TCO modification was well tolerated by the diabody in vitro and in vivo, we selected AVP04-07–TCO(4.7) for further evaluation.

We next evaluated blood clearance and biodistribution in LS174T tumor-bearing mice with AVP04-07–TCO(4.7) and different doses of the ^{177}Lu -radiolabeled tetrazine probe. We found the highest tumor uptake (6.9 %ID/g) and tumor-to-nontumor ratio for 1 Eq of ^{177}Lu -tetrazine (with respect to the diabody), with low kidney uptake leading to a tumor-to-kidney ratio of 5.7. The administered TCO-to-tetrazine ratio of 4.7 was in close agreement with those used in other antibody-based pretargeting strategies, in which investigators observed optimal tumor uptake at tag-to-probe ratios of 5–25 for bispecific monoclonal antibodies or Fab fragment combinations that were used to capture radiolabeled bivalent hapten peptides (36–38). Optimization of the dose for AVP04-07–TCO(4.7) and the time interval between the administration of the diabody and the administration of radiolabeled tetrazine may improve the tumor uptake of radiolabeled tetrazine even further.

Pretargeting biodistribution studies revealed a tumor-to-kidney ratio that was more than 20-fold higher than that previously reported with a radiolabeled DOTA-conjugated AVP04-07 diabody (21), indicating that pretargeting can reduce the kidney radiation dose. Compared with the PEGylation strategy for AVP04-07 (21), the AVP04-07 pretargeting approach resulted in comparable tumor-to-kidney ratios (7.0 vs. 5.7) at 48/50 h after diabody injection, with greater than 4-fold better tumor-to-blood ratio for the pretargeting approach, most likely due to differences in the blood clearance rates for TCO-modified AVP04-07 and PEGylated AVP04-07. In a recent dosimetry study, we found that radiolabeled DOTA–tetrazine (or metabolites) was efficiently eliminated from the kidneys over a time course of several days (24). This may further reduce the kidney dose and favor pretargeting in comparison with direct tumor targeting using radiometal-labeled peptides and proteins.

CONCLUSION

We showed that a pretargeting strategy with a small protein may be an attractive approach for reducing kidney uptake while maintaining high tumor targeting in nuclear imaging and radioimmunotherapy. An effective tumor-targeting agent, an anti-TAG72 diabody that resulted in high kidney uptake when used as a radiometal-labeled analog, showed complete retention of its properties on TCO modification. The administration of radiometal-labeled tetrazine in a second step resulted in efficient tumor uptake and, most importantly, low kidney uptake. This pretargeting strategy could be an important alternative platform, superseding the use of peptides and small proteins as metal–chelate conjugates in imaging and therapy.

DISCLOSURE

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