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CC49 diabody pretargeting using click chemistry in vivo

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ABSTRACT

Radioimmunotherapy and nuclear imaging (Immuno-PET/SPECT) of cancer using radiometal-labeled antibody fragments or peptides is hampered by low tumor-to-kidney ratios due to 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 tumor targeting diabody, AVP04-07, and a low-molecular weight radiolabeled tetrazine probe, which previously showed low kidney retention and relative fast renal clearance. **Methods:** AVP04-07 diabodies were functionalized with TCO tags and in vitro immunoreactivity towards bovine submaxillary mucin and tetrazine reactivity was assessed. Next, pretargeting biodistribution studies were performed in LS174T-tumor bearing mice with AVP04-07-TCO(n) and radiolabeled tetrazine to optimize the TCO modification grade (0 *vs.* 1.8 *vs.* 4.7 TCOs/diabody) and ^{177}Lu-tetrazine dose (0.1 *vs.* 1.0 *vs.* 10 equivalents with respect to diabody). Radiolabeled tetrazine was injected 47h post-diabody injection and mice were euthanized 3h later. A pretargeting SPECT/CT study with ^{111}In-tetrazine was carried out employing the optimized conditions. **Results:** Immunoreactivity for native and TCO-functionalized AVP04-07 was similar and the latter reacted efficiently with radiolabeled tetrazine in vitro. The combination of the pretargeting components AVP04-07 functionalized with 4.7 TCOs and 1 equivalent of ^{177}Lu-tetrazine with respect to diabody showed the most promising biodistribution. Specifically, high ^{177}Lu-tetrazine tumor uptake (6.9 %ID/g) was observed with low renal retention yielding a tumor-to-kidney ratio of 5.7. SPECT/CT imaging confirmed predominant accumulation of radiolabeled tetrazine in tumor with low non-tumor 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 the tumor-homing agent and a fast clearing radioactive probe.

Key words: Diels-Alder, click, pretargeting, diabody

Introduction

Target specific radiolabeled molecules that show rapid tumor targeting with 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 the bone marrow (*1*). Alternatively, low molecular weight proteins (mass <60 kDa) and peptides such as affibodies, nanobodies, single-chain Fv fragments (scFv), or non-covalent stable scFv dimers (diabodies) are rapidly cleared from the blood by the kidneys and can provide better tumor-to-non tumor ratios at shorter time intervals than mAbs, albeit usually combined with a lower target uptake (*2*). However upon glomerular filtration, peptides and antibody fragments are generally reabsorbed by the proximal tubule cells in the kidneys to prevent loss of valuable amino acids, followed by lysosomal degradation (*3*). In case of radiometal-labeled agents, the catabolic ratiometal products have shown prolonged renal entrapment and this typically hampers nuclear imaging and radioimmunotherapy procedures with these agents due to possible nephrotoxicity (*4-10*). Therefore, strategies that reduce 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 have been developed that reduce the renal uptake of radiometal-labeled low-molecular weight agents (*6,11-21*). One example is the conjugation of polyethylene glycol (PEG) to antibody fragments to increase the molecular weight above the renal molecular weight cut-off of 60 kDa resulting in an impressive reduction in kidney uptake (*21,22*). However, due to the reduction in glomerular filtration, these PEGylated antibody fragments showed a relatively long circulation time. Therefore, there is still a need for a general method to use peptide/small proteins for radioimaging/therapy without having to resort to cumbersome and case by case optimization of the structure, while still retaining the fast clearing characteristic of a small molecule. We here focused on the use of an in vivo pretargeting strategy with minimal perturbation of the parent structure and pharmacokinetics (*23*). Pretargeting strategies center on the separate administration of a tumor-binding agent, which binds to a tumor-specific extracellular antigen, and the radionuclide. In the first step, the tumor-binding agent is administered and will accumulate in the tumor. Upon blood clearance of the circulating tumor-binding agent, a fast-clearing radiolabeled probe is administered. The probe efficiently targets the

radioactivity to the prelocalized agent in the tumor, while unreacted probe is rapidly excreted from circulation with low renal retention. We have previously shown that antibody pretargeting through in vivo click chemistry results in superior tumor-to-non tumor ratios compared to conventional radiolabeled mAbs (*24*). In that study, we employed a tumor pretargeting strategy based on the bioorthogonal inverse-electron-demand Diels–Alder reaction between a highly reactive *trans*-cyclooctene (TCO) tagged tumor-homing TAG72-binding antibody and radiometal-labeled tetrazine. Because of the long circulation time of antibodies, clearing agents were injected to effectively remove residual antibody from the blood before administration of radiolabeled tetrazine. The radiolabeled tetrazine displayed a high tumor uptake and low degree of kidney retention and relative fast excretion from the kidney overtime (*24*). We hypothesized that translation of the pretargeting strategy to smaller proteins and peptides may be an attractive approach to overcome the high kidney radiation dose associated with the renal reabsorption of such tumor-homing agents when labeled with a radiometal. In comparison to our previous pretargeting work on long circulating antibodies (*23-25*), the relative fast clearance of such 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 as they provide greater avidity than other agents, including monovalent scFv molecules, and therefore typically show superior tumor uptake (*2,21,22*). In this paper we therefore explored the use of a TAG72-binding diabody AVP04-07 (*21*) as a model construct in a pretargeting approach in LS174T tumor-bearing mice (Figure 1). Results for the diabody pretargeting approach are compared with previously published data for a conventional radiometal-labeled TAG72-binding diabody analog that showed high tumor targeting with fast blood clearance, but which exhibited a high renal retention (*21*).

MATERIALS AND METHODS

General

All reagents and solvents were obtained from commercial sources (Sigma-Aldrich, Acros, Invitrogen, and Merck) and used without further purification unless stated otherwise. ^{111}In-indium chloride, ^{177}Lu-lutetium chloride and sodium ^{125}I-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 and stored at 4°C.

The Bolton-Hunter reagent (N-succinimidyl-3-[4-hydroxyphenyl]propionate, SHPP), gelcode blue protein staining solutions and Zeba desalting spin columns (7 KDa MW cut-off, 0.5 mL) were purchased from Pierce Protein Research (Thermo Fisher Scientific). Mouse serum was purchased from Innovative Research.

The ^{111}In- and ^{177}Lu-labeling yields for 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA)-tetrazine were determined by radio-thin layer chromatography (radio-TLC), using ITLC-SG strips (Varian Inc.) eluted with 200 mM ethylenediaminetetraacetic acid in saline and imaged on a phosphor imager (FLA-7000, Fujifilm). In these conditions, free ^{111}In and ^{177}Lu migrate with R_f = 0.9, while ^{111}In/^{177}Lu-tetrazine remains at the origin. The radiochemical purity of the ^{111}In- and ^{177}Lu-labeled DOTA-tetrazine was determined by radio-high pressure liquid chromatography (radio-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 × 150mm, 5µm), which was 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 ^{125}I-diabody labeling yields were determined with radio-TLC, using ITLC-SG strips eluted with a 1:1 methanol/ethylacetate mixture and imaged on a phosphor imager. In these conditions, free ^{125}I-iodide and ^{125}I-SHPP migrate with R_f = 0.5-0.9, while ^{125}I-diabodies remain at the origin. The radiochemical purity of ^{125}I-AVP04-07 and TCO-conjugated ^{125}I-AVP04-07 were determined by size exclusion chromatography (SEC) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). SEC was carried out on an Agilent 1200 system equipped with a Gabi radioactive detector. The samples were loaded on a Superdex 200 10/300 column (GE Healthcare Life Sciences) 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 using 4-15% PAGE gradient gels (GE Healthcare Life Sciences).

Syntheses of pretargeting components

Synthesis of DOTA-tetrazine (Figure 1B) (*24*), synthesis of the axial isomer of *trans*-cyclooctene-oxymethylbenzamide-NHS (*26*) and AVP04-07 production have been described elsewhere. AVP04-07, a stable ~55 kDa diabody format of monoclonal antibody CC49, was produced by dimerization of scFvs with short 5-residue linkers in a bacterial periplasm fermentation system (*21,26*).

Synthesis of AVP04-07-TCO(n)

1 mg AVP04-07 (6 mg/mL solution in phosphate buffered saline (PBS)) was modified with 2 or 5 molar eq. of the axial isomer of TCO-oxymethylbenzamide-NHS (10 mg/mL in dimethylsulfoxide) in a total volume of 250 µL PBS. The pH was adjusted to 9 with 1M sodium carbonate buffer. The reactions were carried out under agitation for 30 min at room temperature in the dark. Subsequently, the obtained AVP04-07-TCO(n) was purified twice through Zeba desalting spin columns (pre-equilibrated with PBS) and the concentration of the obtained solution was measured by NanoDrop (Thermo Scientific). The TCO loading was revealed from the reaction yield between AVP04-07-TCO(n) (10 µg in 50 µL PBS) and 3 or 7 equivalents of radiolabeled tetrazine (20 min incubation at 37°C, 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 $^{177}LuCl_3$ or $^{111}InCl_3$ in 0.2 M ammonium acetate pH 5.5 and incubated at 60°C for 5 min. The labeling mixture was then mixed with a 10 mM diethylene triamine pentaacetic acid solution (5 µL) and a 20 mg/mL gentisic acid solution in saline at pH 6.5 and incubated for 5 min more. The radiochemical yield and radiochemical purity were assessed by radio-ITLC 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 was 0.07-7 MBq/nmol and ca. 60 MBq/nmol, respectively.

AVP04-07-TCO(n) radiolabeling

Radio-iodination of AVP04-07-TCO(n) diabodies was performed with the Bolton-Hunter method following the manufacturer instructions. Briefly, an adequate amount of sodium ^{125}I-iodide in 50 µL PBS was mixed with 0.1 µg of Bolton-Hunter reagent (SHPP) and 100 µg chloramine-T (N-chloro 4-methylbenzenesulfonamide, sodium salt). The resulting solution was mixed for 10-20 sec, 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 µL 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 under gentle shaking. After incubation, the labeling yields were determined by radio-ITLC. The ^{125}I-labeled diabodies were purified twice through Zeba spin desalting columns according to manufacturer instructions (pre-equilibrated with saline) and the radiochemical purities were 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/µg by adding cold AVP04-07 and sterile saline.

Immunoreactivity

Iodine-125-labeled AVP04-07 and AVP04-07-TCO(n) were reacted with 20 eq. bovine submaxillary mucin (BSM, a surrogate target antigen for human TAG72) in 1% bovine serum albumin in PBS at 37°C for 20 min (350 rpm). The reaction mixtures where then analyzed by SEC to identify and quantify diabody and diabody-BSM complexes.

Animal experiments

All animal experiments were performed according to the principles of laboratory animal care (NIH publication 85–23, revised 1985) and the Dutch national law “Wet op de Dierproeven” (Stb 1985, 336). The in vivo experiments were performed in tumor-bearing nude female Balb/C mice (20-25 g body weight, Charles River Laboratories). The human colon cancer cell line LS174T was obtained from the ATCC and maintained in Eagle’s minimal essential medium (Sigma) supplemented with 10% heat inactivated fetal calf serum (Gibco), penicillin (100 U/mL), streptomycin (100 µg/mL) and 2 mM Glutamax. Mice were inoculated subcutaneously with 5 × 10^6 cells in 100 µL sterile PBS and were used 7-10 days after tumor inoculation, when the tumors reached an approximately 70-200 mm^3 size. 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 PBS. The sample radioactivity was counted in a gamma counter (Wizard 1480, PerkinElmer) along with standards to determine the percent injected dose per gram (%ID/g) and the percent injected dose per organ (%ID/organ). The tissues from dual-isotope experiments were measured using dual-isotope protocol (10-80 keV and 155-380 keV energy windows 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 µg/100 µL per mouse, ca. 0.2 MBq). The mice were serially bled at 5, 30 min, 1, 2, 6, and 24h. Subsequently, the mice were injected with 6.7 nmol ^{177}Lu-tetrazine (8.52 µg/80 µL per mouse containing 100 µg gentisic acid; ca. 0.5 MBq) at 47 h post-diabody injection and euthanized 3 h post-tetrazine injection, after which organs and tissues of interest were harvested. Two other groups of 4 LS174T tumor-bearing mice were injected intravenously with ^{125}I-AVP04-07-TCO(4.7) (35 µg/100 µL per mouse, ca. 0.2 MBq) and 0.67 nmol or 67 pmol ^{177}Lu-tetrazine was administered at 47 h post-diabody injection. The mice were euthanized 3 h post-tetrazine injection, after which organs and tissues of interest were harvested. Blood samples, organs, and tissues were counted for radioactivity. Blood clearance data was fitted with a two-phase exponential decay function and area-under-the-curve (AUC) was determined. Blood half-lives subsequently were derived using formula $t_{1/2,AUC} = \ln(2) \times AUC/C_0$, with C_0 representing the probe concentration (%ID/g) in blood at t = 0.

SPECT/CT imaging experiment

One LS174T tumor-bearing mouse was injected with AVP04-07-TCO(4.7) (35 µg/100 µL) and ^{111}In-tetrazine (0.67 nmol/80 µL containing 100 µg gentisic acid; ca. 42 MBq) following the pretargeting protocol. Approximately 90 min post-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-pinhole collimators (pinhole diameter: 1.4 mm). First a CT scan (180 projections; 1000 msec per projection; 45 kV peak tube voltage; 177 mA tube current; 35 mm field of view) and then a SPECT scan (24 projections; 120 sec per projection; photopeaks for ^{111}In set to 171 keV (15% FW) and 245 keV (20% FW)) were performed. Three hours post-tetrazine injection the mouse was euthanized by anesthesia overdose and a high resolution SPECT/CT scan was performed (360 projections and 2000 msec per projection for CT; 36 projections and 300 sec per projection for SPECT). The SPECT image was reconstructed using HiSPECTNG (SciVis GMBH) to an isotropic voxel size of 300 µm. The CT image was reconstructed using InVivoScope (Bioscan) to an isotropic voxel size of 200 µm.

Data analysis

The data are presented as mean %ID/g or %ID/organ ± one standard deviation (SD). Standard one-way ANOVA with Bonferroni's post-hoc testing for multiple group comparisons, curve fitting, and calculation of AUC were performed with GraphPad Prism version v 4.1. The difference between two data points was considered statistically significant when $P<0.05$.

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) with the aim to afford two distinct modification ratios within the range of ca. 2-5 TCO groups per diabody. Radioiodination of AVP04-07 and AVP04-07-TCO was carried out 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 >98%, as confirmed by radio-TLC and SDS-PAGE analysis (Supplemental Figs. 2,3). DOTA-tetrazine was labeled with ^{177}Lu for in vitro reactivity assays and biodistribution experiments or ^{111}In for SPECT/CT imaging with >99% labeling yield and >95% radiochemical purity (Supplemental Figs. 3,4). The reaction yield between AVP04-07-TCO(n) and 3 or 7 equivalents of radiolabeled tetrazine indicated the presence of on average 1.8 and 4.7 TCO groups per diabody. In an immunoreactivity assay, iodinated AVP04-07-TCO showed quantitative binding to 20 equivalents of TAG72-positive BSM (Supplemental Fig. 5).

Optimization of diabody TCO loading for in vivo pretargeting experiments

To determine the optimal diabody TCO loading, three ^{125}I-labeled AVP04-07 constructs containing respectively 0, 1.8, and 4.7 TCOs/diabody were injected in LS174T tumor-bearing mice. Blood clearance data showed a 1.7-fold and 1.9-fold slower blood clearance for respectively ^{125}I-AVP04-07-TCO(1.8) and ^{125}I-AVP04-07-TCO(4.7) compared with native ^{125}I-AVP04-07 (Figure 2). All diabodies had essentially cleared from circulation at 50h post-diabody injection. At 47h post-diabody injection, 10 equivalents of ^{177}Lu-tetrazine (with respect to diabody) was administered by intravenous injection. Dual-isotope biodistribution

at 3h post-^{177}Lu-tetrazine injection, corresponding to 50h post-diabody injection showed similar results for the three diabodies (Figure 3A, Supplemental Table 1). Specifically, the ^{125}I-diabodies showed high tumor uptake (12-14 %ID/g), while 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 ^{125}I-levels in stomachs and thyroids indicated there was no significant in vivo dehalogenation of the ^{125}I-AVP04-07 constructs.

Administration of 10 equivalents of ^{177}Lu-tetrazine led to low tumor uptake for mice pretargeted with native AVP04-07, while for AVP04-07-TCO(1.8) and AVP04-07-TCO(4.7) the ^{177}Lu-tetrazine tumor levels were significantly higher and correlated with the TCO concentration, affording respectively 0.78 ± 0.20 and 1.55 ± 0.22 %ID/g (Supplemental Table 1). Calculation of the on-tumor reaction yield (based on TCO) between the ^{125}I-labeled diabody-TCO constructs and ^{177}Lu-tetrazine revealed 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 ^{177}Lu-tetrazine retention was low to very low and independent of the pre-administered diabody. As the tumor-to-non tumor ratios for ^{177}Lu-tetrazine in mice pretargeted with the AVP04-07-TCO(4.7) were significantly higher for most tissues compared with AVP04-07-TCO(1.8) (Supplemental Table 2), the former was selected for further evaluation.

Optimization of ^{177}Lu-tetrazine dose to improve tumor uptake together with SPECT/CT imaging

To investigate whether tumor uptake and the ratios of tumor-to-blood/normal tissue could be further improved, we investigated two additional dose levels. Pretargeting biodistribution studies with 1.0 and 0.1 equivalent ^{177}Lu-tetrazine (with respect to diabody), administered at 47h post-diabody injection, were performed in mice pretargeted with ^{125}I-AVP04-07-TCO(4.7) (Figure 4A, Supplemental Table 3). While the tumor-to-blood ratio decreased with decreasing amount of injected ^{177}Lu-tetrazine (Figure 4B) most other tumor-to-organ ratios were significantly higher for 1 equivalent ^{177}Lu-tetrazine administered with respect to AVP04-07-TCO(4.7) compared to either 0.1 or 10 equivalents (Supplemental Table 4). Specifically, the tumor-to-kidney ratio increased from 1.2 to 5.7 when administering 1 instead of 10 equivalents of ^{177}Lu-tetrazine. In addition, the tumor uptake was maximal at that dose affording 6.9 ± 1.1 %ID/g. Therefore, 1 equivalent of ^{177}Lu-tetrazine with respect to diabody was considered to be the optimal ^{177}Lu-tetrazine dose. A subsequent SPECT/CT imaging experiment using the pretargeting protocol at this optimal dose confirmed

high ^{111}In-tetrazine uptake in tumor and low retention in non-target organs (Figure 5). This was expected as we and others earlier demonstrated similar biodistribution of ^{177}Lu and ^{111}In-labeled mAbs and small molecular weight agents, employing DOTA as chelator. (*25,27-30*).

Discussion

The tubular reabsorption and subsequent high renal retention of radiometal-labeled peptides, antibody fragments and other small proteins hampers their use in nuclear imaging and targeted radiotherapy of cancer due to possible nephrotoxicity. Therefore, various strategies have been developed that aim for a reduction in renal uptake of such radiometal-labeled low-molecular weight cancer-targeting agents. Co-administration 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*). The co-infusion of the plasma expander Gelofusine (with or without the co-administration of cationic amino acids) also showed strong reduction of kidney uptake for a radiolabeled octreotate (*31*) and a radiolabeled nanobody (*28,29*). In another approach, radiolabeled chelates were designed to be cleaved off from the protein/peptide upon glomerular filtration but before reabsorption, allowing renal excretion of the radiolabel. This strategy showed a 75% reduction in renal uptake of the radiolabeled chelate (*14*). Importantly, none of the above approaches could completely inhibit the renal reabsorption of small radiolabeled agents and for the approaches that have been evaluated in tumor-bearing animals the kidney retention was still higher than the tumor uptake (*14,32,33*). Therefore, more efficient strategies are needed to reduce radiation toxicity in kidney. 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 affibodies with albumin-binding domains resulted in a strong reduction in glomerular filtration and a 7-fold higher tumor uptake compared to kidney uptake (in %ID/g) (*15,21*). These strategies however are hampered by a prolonged residence time of the agents in blood resulting in relative low tumor-to-blood ratios and risks for radiation toxicity to the bone marrow, similar to intact antibodies. We previously developed a click-chemistry pretargeting strategy that may be an attractive alternative to the above reengineering (*24*). To demonstrate this, an anti-TAG72 diabody, also known as AVP04-07, was selected as low-molecular weight tumor-homing model construct.

Previous studies have shown that while a radiolabeled DOTA-conjugate of this diabody efficiently targeted the tumor in LS174T tumor-bearing mice, the renal retention of the radiometal label was very high (*21*) resulting in a problematic tumor-to-kidney ratio of 0.25 at 48h post-injection. PEGylation of the AVP04-07 diabody diminished the kidney uptake and increased blood circulation, boosting the 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 48 h post-injection was 4.4 versus >50 for native AVP04-07. We set out to develop a strategy that affords a practical tumor-to-kidney ratio of > 4 while maintaining the high tumor-to-blood ratios typical of small molecular weight agents such as diabodies. We applied our previously developed click-chemistry pretargeting approach to the AVP04-07-diabody, employing *trans*-cyclooctene (TCO)-modified AVP04-07 diabody and radiolabeled tetrazine. The diabody constructs, comprising 1.8 and 4.7 TCO per diabody, retained their immunoreactivity towards BSM when compared to the unmodified diabody, demonstrating that the small TCO tag when bound to surface lysine residues is well-tolerated by the diabody. In addition, the diabody-TCO constructs reacted efficiently with the radiolabeled ^{177}Lu-tetrazine probe in PBS. In mice, the different radio-iodinated diabodies (^{125}I-AVP04-07-TCO(n)) had a comparable biodistribution, indicating that the modification of the diabody with TCO tags did not significantly perturb its pharmacokinetics. We observed a relatively minor increase in blood clearance half-life for the TCO-modified diabodies compared to the native diabody, which is most likely due to non-covalent and/or covalent albumin binding (*34,35*). The similar tumor uptake for native and tagged diabody 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. Following administration of radiolabeled ^{177}Lu-tetrazine and dual isotope biodistribution, we found on-tumor reaction yields (based on TCO) of 31.2 ± 7.2 % and 37.7 ± 3.1 % for AVP04-07-TCO(1.8) and AVP04-07-TCO(4.7), which corresponds well to the earlier found 46 % in a pretargeting study with the same molar dose of full CC49 mAb modified with 8 TCOs (*24*). Because ^{177}Lu-tetrazine showed the highest tumor accumulation in AVP04-07-TCO(4.7) pretargeted animals 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 then evaluated blood clearance and biodistribution in LS174T-tumor bearing mice with AVP04-07-TCO(4.7) and different doses of radiolabeled ^{177}Lu-tetrazine probe. We found the highest tumor uptake (6.9

%ID/g) and tumor-to-non tumor ratios for 1 eq ^{177}Lu-tetrazine with respect to diabody, with low kidney uptake leading to a tumor-to-kidney ratio of 5.7. The administered TCO:tetrazine ratio of 4.7 is in close agreement with other antibody-based pretargeting strategies that observed optimal tumor uptake at tag-probe ratios of 5 to 25 for bispecific monoclonal antibodies or Fab fragment combinations which were used to capture radiolabeled bivalent hapten peptides (*36*-*38*). Optimization of the dose for AVP04-07-TCO(4.7) and time interval between the administration of diabody and radiolabeled tetrazine may even further improve tumor uptake of the radiolabeled tetrazine.

The pretargeting biodistribution studies afforded a >20-fold increased tumor-to-kidney ratio compared to the previously reported radiolabeled DOTA-conjugated AVP04-07 diabody (*21*), indicating that pretargeting can reduce the kidney radiation dose. Compared to the PEGylation strategy for AVP04-07 (*21*), the results for the AVP04-07 pretargeting approach afforded comparable tumor-to-kidney ratios (7.0 *vs.* 5.7) at 48/50 h post diabody injection, with a >4-fold better tumor-to-blood ratio for the pretargeting approach, most likely due to differences in the blood clearance rate for TCO-modified AVP04-07 and PEGylated AVP04-07. In a recent dosimetry study we found that radiolabeled DOTA-tetrazine (or metabolites) is 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 here show that a pretargeting strategy with a small protein may be an attractive approach in nuclear imaging and radioimmunotherapy to reduce kidney uptake while maintaining high tumor targeting. An effective tumor-targeting agent, a TAG72 diabody that had a high kidney uptake as a directly radiometal-labeled analog, showed complete retention of its properties upon TCO-modification. Administration of a radiometal-labeled tetrazine in a second step, showed 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 for imaging and therapy.

Disclosure

All authors declare a conflict of interest due to their employment at respectively Tagworks Pharmaceuticals, Philips Research, and Avipep Pty Ltd.

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References

1. Boswell CA, Brechbiel MW. Development of radioimmunotherapeutic and diagnostic antibodies: an inside-out view. *Nucl Med Biol.* 2007;34(7):757-778.

2. Olafsen T, Elgqvist J, Wu AM. Protein targeting constructs in alpha therapy. Curr Radiopharm. 2011; 4(3):197-213.

3. Maack T, Johnson V, Kan ST, Figueiredo J, Sigulem D. Renal filtration, transport, and metabolism of low molecular weight proteins: a review. *Kidney Int.* 1979;16(3):251-270.

4. Malmberg J, Sandström M, Wester K, Tolmachev V, Orlova A. Comparative biodistribution of imaging agents for in vivo molecular profiling of disseminated prostate cancer in mice bearing prostate cancer xenografts: focus on ^{111}In- and ^{125}I-labeled anti-HER2 humanized monoclonal trastuzumab and ABY-025 affibody. *Nucl Med Biol.* 2011;38(8):1093-1102.

5. Behr TM, Goldenberg DM, Becker W. Reducing the renal uptake of radiolabeled antibody fragments and peptides for diagnosis and therapy: present status, future prospects and limitations. *Eur J Nucl Med.* 1998;25(2):201-212.

6. Williams LE, Wu AM, Kenanova VE, Olafsen T, Yazaki PJ. Numerical comparison of iodine-based and indium-based antibody biodistributions. *Cancer Biother Radiopharm*. 2014 29(3):91-98.

7. Jin ZH, Furukawa T, Claron M, Boturyn D, Claron M, Boturyn D, Coll JL, Fukumura T, Fujibayashi Y, Dumy Y, Saga T. Positron emission tomography imaging of tumor angiogenesis and monitoring of antiangiogenic efficacy using the novel tetrameric peptide probe ^{64}Cu-cyclam-RAFT-c(-RGDfK-)$_4$. *Angiogenesis.* 2012;15(4):569-580.

8. Dalmo J, Rudqvist N, Spetz J, Laverman P, Nilsson O, Ahlman H, Forssell-Aronsson E. Biodistribution of ^{177}Lu-octreotate and ^{111}In-minigastrin in female nude mice transplanted with human medullary thyroid carcinoma GOT2. *Oncol Rep.* 2012;27(1):174-181.

9. de Jong M, Krenning E. New advances in peptide receptor radionuclide therapy. *J Nucl Med.* 2002;43(5):617-620.

10. Movahedi K, Schoonooghe S, Laoui D, Houbracken I, Waelput W, Breckpot K, Bouwens L, Lahoutte T, De Baetselier P, Raes G, Devoogdt N, Van Ginderachter JA. Nanobody-based targeting of the

macrophage mannose receptor for effective in vivo imaging of tumor-associated macrophages. *Cancer Res.* 2012;72(16):4165-4177.

11. Rolleman EJ, Valkema R, de Jong M, Kooij PP, Krenning EP. Safe and effective inhibition of renal uptake of radiolabelled octreotide by a combination of lysine and arginine. *Eur J Nucl Med Mol Imaging.* 2003;30(1):9-15.

12. Behr TM, Becker WS, Sharkey RM, Juweid ME, Dunn RM, Bair HJ, Wolf FG, Goldenberg DM. Reduction of renal uptake of monoclonal antibody fragments by amino acid infusion. *J Nucl Med.* 1996;37(5):829–833.

13. Kim I, Kobayashi H, Yoo TM, Kim MK, Le N, Han ES, Wang QC, Pastan I, Carrasquillo JA, Paik CH. Lowering of pI by acylation improves the renal uptake of ^{99m}Tc-labeled anti-Tac dsFv: effect of different acylating reagents. *Nucl Med Biol.* 2002;29(8):795–801.

14. Li L, Olafsen T, Anderson AL, Wu A, Raubitschek AA, Shively JE. Reduction of kidney uptake in radiometal labeled peptide linkers conjugated to recombinant antibody fragments: site-specific conjugation of DOTA-peptides to a Cys-diabody. *Bioconjug Chem.* 2002;13(5):985–995.

15. Orlova A, Jonsson A, Rosik D, Lundqvist H, Lindborg M, Abrahmsen L, Ekblad C, Frejd FY, Tolmachev V. Site-specific radiometal labeling improved biodistribution using ABY-027, a novel HER2-targeting affibody molecule-albumin binding domain fusion protein. *J Nucl Med.* 2013;54(6):961-968.

16. Li L, Yazaki PJ, Anderson AL, Crow D, Colcher D, Wu AM, Williams LE, Wong JY, Raubitschek A, Shively JE. Improved biodistribution and radioimmunoimaging with poly(ethylene glycol)-DOTA-conjugated anti-CEA diabody. *Bioconjug Chem.* 2006;17(1):68-76.

17. Vegt E, Eek A, Oyen WJ, de Jong M, Gotthardt M, Boerman OC. Albumin-derived peptides efficiently reduce renal uptake of radiolabelled peptides. *Eur J Nucl Med Mol Imaging.* 2010;37(2):226-234.

18. Béhé M, Kluge G, Becker W, Gotthardt M, Behr TM. Use of polyglutamic acids to reduce uptake of radiometal-labeled minigastrin in the kidneys. *J Nucl Med.* 2005; 46(6):1012–1015.

19. Rolleman EJ, Krenning EP, van Gameren A, Bernard BF, de Jong M. Uptake of [(111)In-DTPA(0)]octreotide in the rat kidney is inhibited by colchicine and not by fructose. *J Nucl Med.* 2004;45(4):709–713.

20. Gotthardt M, van Eerd-Vismale J, Oyen WJ, de Jong M, Zhang H, Rolleman E, Maecke HR, Béhé M, Boerman O. Indication for different mechanisms of kidney uptake. *J Nucl Med.* 2007;48(4):596-601.

21. Li L, Turatti F, Crow D, Bading JR, Anderson AL, Poku E, Yazaki PJ, Williams LE, Tamvakis D, Sanders P, Leong D, Raubitschek A, Hudson PJ, Colcher D, Shively JE. Monodispersed DOTA-PEG-conjugated anti-TAG-72 diabody has low kidney uptake and high tumor-to-blood ratios resulting in improved ^{64}Cu PET. *J Nucl Med.* 2010;51(7):1139-1146.

22. Li L, Crow D, Turatti F, Bading JR, Anderson AL, Poku E, Yazaki PJ, Carmichael J, Leong D, Wheatcroft D, Raubitschek AA, Hudson PJ, Colcher D, Shively JE. Site-specific conjugation of monodispersed DOTA-PEGn to a thiolated diabody reveals the effect of increasing PEG size on kidney clearance and tumor uptake with improved 64-copper PET imaging. *Bioconjug Chem.* 2011;22(4):709-716.

23. Rossin R, Renart Verkerk P, van den Bosch SM, Vulders RCM, Verel I, Lub J, Robillard MS. In vivo chemistry for pretargeted tumor imaging in live mice. *Angew Chem Int Ed.* 2010;49(19):3375-3378.

24. Rossin R, Läppchen T, van den Bosch SM, Laforest R, Robillard MS. Diels-Alder reaction for tumor pretargeting: in vivo chemistry can boost tumor radiation dose compared to directly labeled antibody. *J Nucl Med.* 2013;54(11):1989-1995.

25. Rossin R, van den Bosch SM, ten Hoeve W, Carvelli M, Versteegen RM, Lub J, Robillard MS. Highly reactive *trans*-cyclooctene tags with improved stability for Diels-Alder chemistry in living systems. *Bioconjug Chem.* 2013;24(7):1210-1217.

26. Powers GA, Hudson PJ, Wheatcroft MP. Design and production of multimeric antibody fragments, focused on diabodies with enhanced clinical efficacy. *Methods Mol Biol.* 2012;907:699-712.

27. Zhang H, Chen J, Waldherr C, Hinni K, Waser B, Reubi JC, Maecke HR. Synthesis and evaluation of bombesin derivatives on the basis of pan-bombesin peptides labeled with indium-111, lutetium-

177, and yttrium-90 for targeting bombesin receptor-expressing tumors. Cancer Res 2004;64(18):6707-6715.

28. Vallabhajosula S, Kuji I, Hamacher KA, Konishi S, Kostakoglu L, Kothari PA, Milowski MI, Nanus DM, Bander NH, Goldsmith SJ. Pharmacokinetics and biodistribution of 111In- and 177Lu-labeled J591 antibody specific for prostate-specific membrane antigen: prediction of 90Y-J591 radiation dosimetry based on 111In or 177Lu? *J Nucl Med.* 2005;46(4):634-641.

29. Mohsin H, Fitzsimmons J, Shelton T, Hoffman TJ, Cutler CS, Lewis MR, Athey PS, Gulyas G, Kiefer GE, Frank RK, Simon J, Lever SZ, Jurisson SS. Preparation and biological evaluation of 111In-177Lu- and 90Y-labeled DOTA analogues conjugated to B72.3. *Nucl Med Biol.* 2007;34(5):493-502.

30. Rossin R, van Duijnhoven SMJ, Läppchen T, van den Bosch SM, Robillard MS. Trans-cyclooctene tag with improved properties for tumor pretargeting with the Diels−Alder reaction. *Mol Pharm.* 2014;11(9):3090-3096.

31. Melis M, Bijster M, de Visser M, Konijnenberg MW, de Swart J, Rolleman EJ, Boerman OC, Krenning EP, de Jong M. Dose-response effect of Gelofusine on renal uptake and retention of radiolabelled octreotate in rats with CA20948 tumours. *Eur J Nucl Med Mol Imaging.* 2009;36(12):1968-1976.

32. Gainkam LO, Caveliers V, Devoogdt N, Vanhove C, Xavier C, Boerman O, Muyldermans S, Bossuyt A, Lahoutte T. Localization mechanism and reduction of renal retention of technetium-99m labeled epidermal growth factor receptor-specific nanobody in mice. *Contrast Media Mol Imaging.* 2011;6(2):85-92.

33. D'Huyvetter M, Vincke C, Xavier C, Aerts A, Impens N, Baatout S, De Raeve H, Muyldermans S, Caveliers V, Devoogdt N, Lahoutte T. Targeted radionuclide therapy with a ^{177}Lu-labeled anti-HER2 nanobody. *Theranostics.* 2014;4(7):708-720.

34. van den Bosch SM, Rossin R, Renart Verkerk P, ten Hoeve W, Janssen HM, Lub J, Robillard MS. Evaluation of strained alkynes for Cu-free click reaction in live mice. *Nucl Med Biol.* 2013;40(3):415-423.

35. Poole TH, Reisz JA, Zhao W, Poole LB, Furdui CM, King SB. Strained cycloalkynes as new protein sulfenic acid traps. *J Am Chem Soc.* 2014;136(17):6167-6170.

36. Sharkey RM, Cardillo TM, Rossi EA, Chang C, Karacay H, McBride WJ, Hansen HJ, Horak ID, Goldenberg DM. Signal amplification in molecular imaging by pretargeting a multivalent, bispecific antibody. *Nat Med.* 2005;11(11):1250-1255.

37. Karacay H, Brard Y, Sharkey RM, Chang C, Rossi EA, McBride WJ, Ragland DR, Horak ID, Goldenberg DM. Therapeutic advantage of pretargeted radioimmunotherapy using a recombinant bispecific antibody in a human colon cancer xenograft. *Clin Cancer Res.* 2005;11(21):7879-7885.

38. van Rij CM, Frielink C, Goldenberg DM, Sharkey RM, Franssen GM, Lütje S, McBride WJ, Oyen WJ, Boerman OC. Pretargeted immunoPET of prostate cancer with an anti-TROP-2 x anti-HSG bispecific antibody in mice with PC3 xenografts. *Mol Imaging Biol.* 2015 17(1):94-101.

Figures

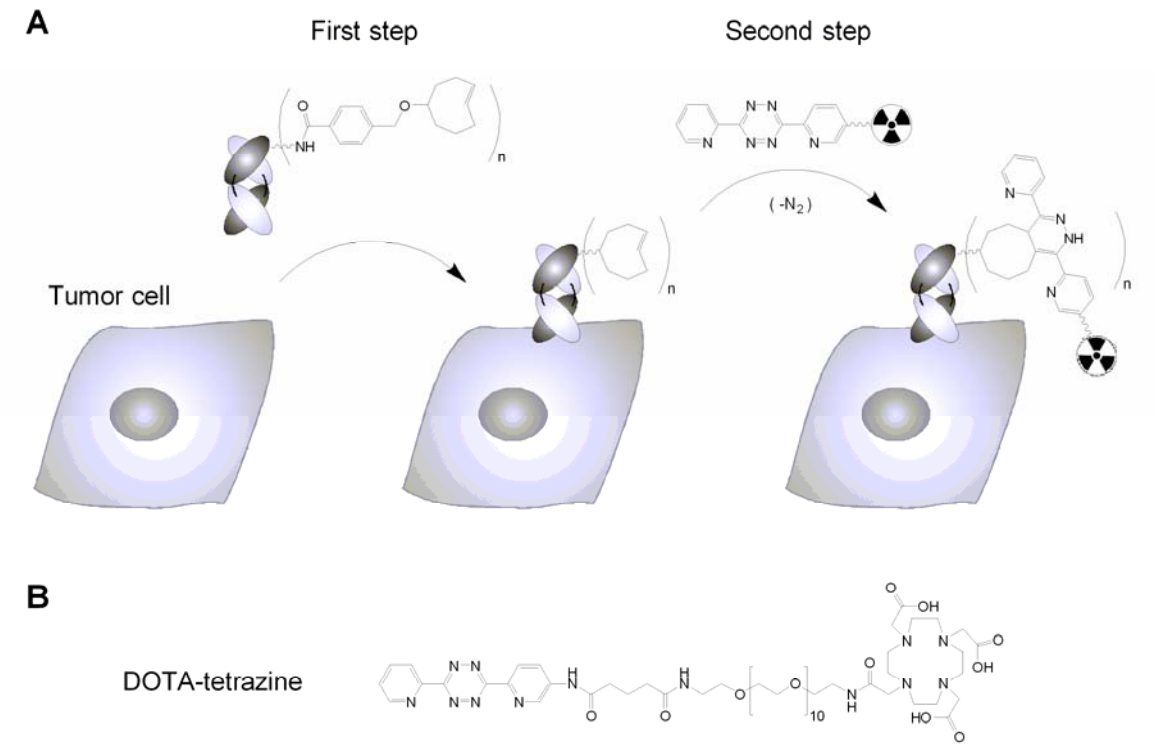

Figure 1: (A) Schematic of diabody tumor pretargeting using the inverse-electron-demand Diels–Alder reaction between a *trans*-cyclooctene tagged diabody and radiolabeled tetrazine. Upon tumor binding and blood clearance of the diabody, administration of radiolabeled tetrazine targets the radioactivity to the prelocalized diabody in the tumor. (B) Molecular structure of DOTA-tetrazine.

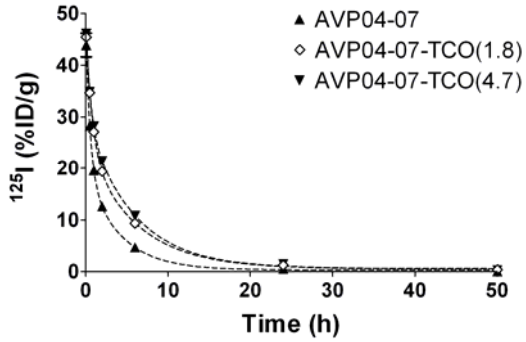

Figure 2: Blood clearance of 35 µg ^{125}I-AVP04-07, ^{125}I-AVP04-07-TCO(1.8), and ^{125}I-AVP04-07-TCO(4.7) in LS174T tumor-bearing mice. Data points represent the mean %ID/g ± SD (n = 3). Half lives $t_{1/2,AUC}$ are 1.92 h for ^{125}I-AVP04-07, 3.30 h for ^{125}I-AVP04-07-TCO(1.8), and 3.58 h for ^{125}I-AVP04-07-TCO(4.7).

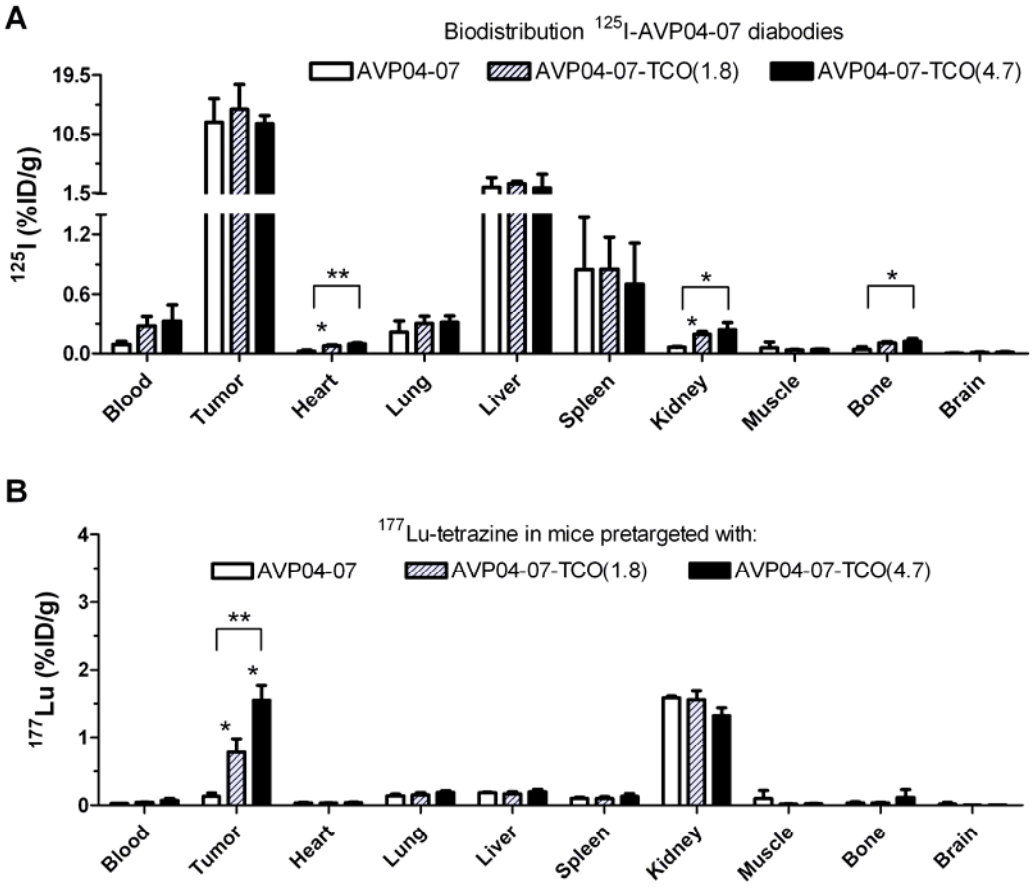

Figure 3: Dual-isotope biodistribution for pretargeting with ^{125}I-AVP04-07 or, ^{125}I-AVP04-07-TCO(1.8), or ^{125}I-AVP04-07-TCO(4.7), and ^{177}Lu-tetrazine in LS174T tumor-bearing mice. The mice were injected with one of the ^{125}I-AVP04-07 diabodies, followed by administration of ^{177}Lu-tetrazine (10 eq. with respect to diabody) at 47 h post-diabody injection. The mice were euthanized 3 h later. Panel A and B show the biodistribution for the ^{125}I-AVP04-07 diabodies and ^{177}Lu-tetrazine, respectively. The bars represent the mean %ID/g ± SD (n = 3; * P<0.05, ** P<0.001).

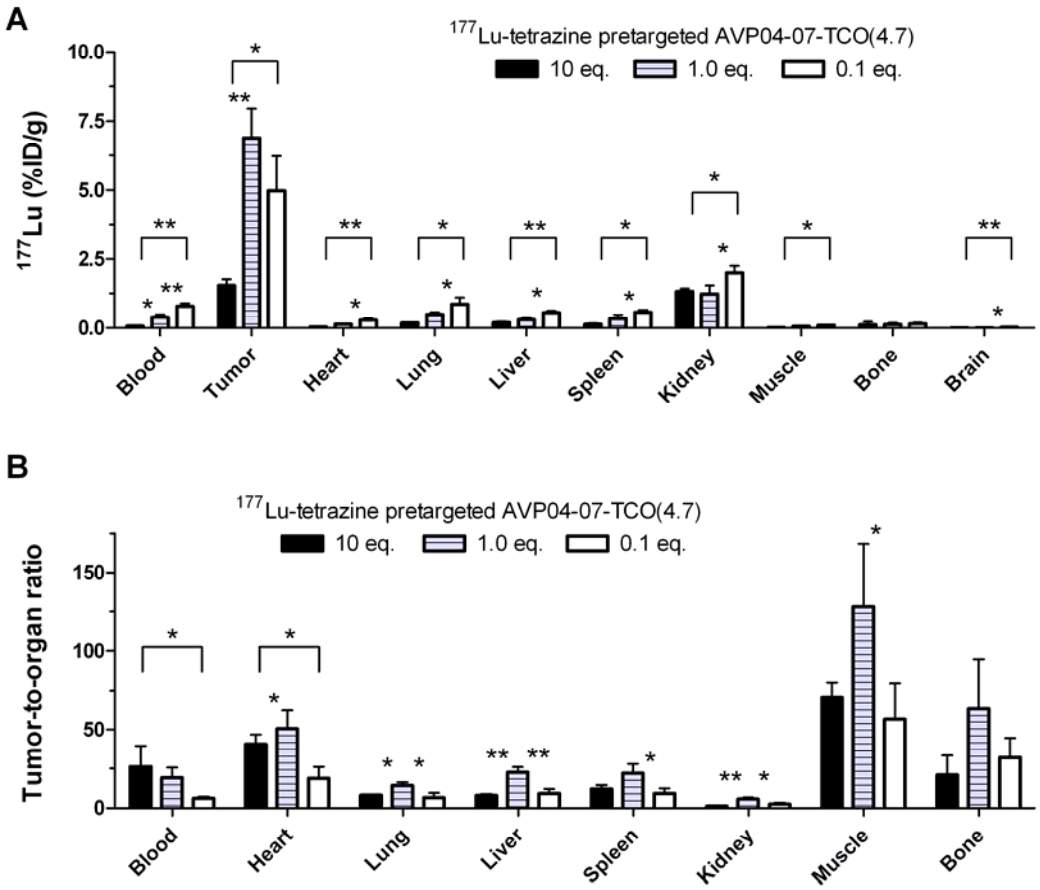

Figure 4: ^{177}Lu-tetrazine biodistribution in LS174T tumor-bearing mice pretargeted with AVP04-07-TCO(4.7). The mice were injected with AVP04-07-TCO(4.7), followed by administration of ^{177}Lu-tetrazine (10 eq. (n = 3), 1.0 eq., or 0.1 eq. (n = 4) with respect to diabody) at 47 h post-diabody injection. The mice were euthanized 3 h later. Panel A and B show the biodistribution and tumor-to-organ ratios for ^{177}Lu-tetrazine, respectively. The bars represent the mean ± SD (*P<0.05, **P<0.001).

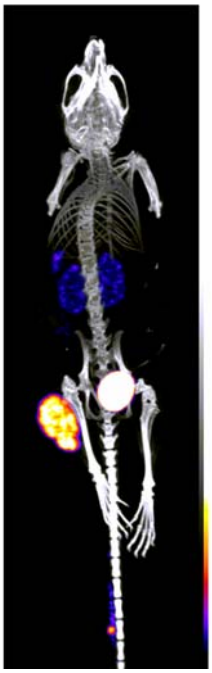

Figure 5: SPECT/CT image (maximum intensity projection) of ^{111}In-tetrazine in a LS174T tumor-bearing mouse pretargeted with AVP04-07-TCO(4.7). The image shows high radioactivity uptake in the tumor (hind limb) and bladder (urine). Low retention of radioactivity is also observed in the kidney, while all other organs and tissues showed negligible levels of radioactivity.