Video Article

Preparation and Evaluation of ^{99m}Tc-labeled Tridentate Chelates for Pre-targeting Using Bioorthogonal Chemistry

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Abstract

Pre-targeting combined with bioorthogonal chemistry is emerging as an effective way to create new radiopharmaceuticals. Of the methods available, the inverse electron demand Diels-Alder (IEDDA) cycloaddition between a radiolabeled tetrazines and *trans*-cyclooctene (TCO) linked to a biomolecule has proven to be a highly effective bioorthogonal approach to imaging specific biological targets. Despite the fact that technetium-99m remains the most widely used isotope in diagnostic nuclear medicine, there is a scarcity of methods for preparing ^{99m}Tc-labeled tetrazines. Herein we report the preparation of a family of tridentate-chelate-tetrazine derivatives and their Tc(I) complexes. These hitherto unknown compounds were radiolabeled with ^{99m}Tc using a microwave-assisted method in 31% to 83% radiochemical yield. The products are stable in saline and PBS and react rapidly with TCO derivatives *in vitro*. Their *in vivo* pre-targeting abilities were demonstrated using a TCO-bisphosphonate (TCO-BP) derivative that localizes to regions of active bone metabolism or injury. In murine studies, the ^{99m}Tc-tetrazines showed high activity concentrations in knees and shoulder joints, which was not observed when experiments were performed in the absence of TCO-BP. The overall uptake in non-target organs and pharmacokinetics varied greatly depending on the nature of the linker and polarity of the chelate.

Video Link

The video component of this article can be found at https://www.jove.com/video/55188/

Introduction

^{99m}Tc remains the dominant radioisotope used in diagnostic nuclear medicine, with over 50 million imaging procedures conducted per year worldwide[1,2,3]. The majority of ^{99m}Tc agents used clinically are perfusion type radiopharmaceuticals. There are a limited number of actively targeted compounds in which ^{99m}Tc is directed to bind a specific biomarker through ligation to a targeting construct. The creation of targeted ^{99m}Tc radiopharmaceuticals is often hindered by the influence of ^{99m}Tc-ligand complexes on the ability of the targeting molecule to bind the biomarker of interest, or the isotopes half-life is not long enough for use with higher molecular weight biomolecules such as antibodies. The latter typically requires several days before images are acquired in order for the biomolecule to clear from non-target tissues. Pre-targeting offers an alternative approach to overcome these challenges.

Pre-targeting combined with bioorthogonal chemistry has been shown to be an effective way to develop new molecular imaging probes for both fluorescence and radio-imaging[4,5,6,7,8]. The inverse electron demand Diels-alder (IEDDA) reaction between 1,2,4,5-tetrazine (Tz) and *trans*-cyclooctene (TCO) derivatives, as shown in **Figure 1**, has been shown to be particularly effective[6]. The IEDDA reaction with these components can exhibit fast kinetics in PBS ($k_2 \approx 6{,}000\ M^{-1}\ s^{-1}$) and high selectivity, making it ideal for *in vivo* pre-targeting applications[9,10].

The most common approach used involves administering a TCO-derived targeting vector and following a sufficient delay period, a radiolabeled tetrazine is administered. Radiolabeled tetrazines based on ^{11}C, ^{18}F, ^{64}Cu, ^{89}Zr, and ^{111}In have been reported[11,12,13,14,15]. In contrast, there is only one report of a ^{99m}Tc-labeled Tz, which was prepared using a HYNIC type ligand requiring the use of co-ligands to prevent protein binding and degradation *in vivo*[16]. As an alternative, we report here the synthesis of ^{99m}Tc(I) labeled tetrazines using a family of ligands which form stable tridentate complexes with a $[^{99m}Tc(CO)_3]^+$ core.

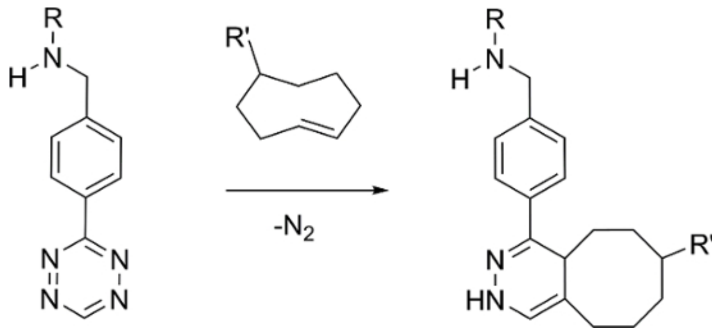

Figure 1: The bioorthogonal IEDDA reaction between tetrazine and *trans*-cyclooctene. Please click here to view a larger version of this figure.

The family of ligands prepared contain tridentate chelates that vary in polarity and the nature of the linker group between the metal binding region and the Tz (**Figure 2**). The goal was to identify a ^{99m}Tc-Tetrazine construct that could effectively localize and react with TCO-labeled sites *in vivo* and rapidly clear when not bound, in order to yield high target-to-non-target ratios. To test the ligands, a TCO-derivative of a bisphosphonate (TCO-BP) was used[17]. We have shown previously that TCO-BP localizes to areas of active bone metabolism and can react with radiolabeled tetrazines *in vivo*[18]. It is a convenient reagent to test new tetrazines, because it can be prepared in a single step and experiments can be performed in normal mice where localization occurs primarily in the joints (knees and shoulders).

Protocol

Animal studies were approved by the Animal Research Ethics Board at McMaster University in accordance with Canadian Council on Animal Care (CCAC) guidelines.

1. Radiolabeling of Tz-tridentate Ligands with ^{99m}Tc

CAUTION: The following procedures require the use of radioactive compounds. Work should only be done in a licensed laboratory with adherence to safety and disposal regulations. Microwave reactions should be performed in a microwave specifically designed for chemical synthesis.

1. **Synthesis of [^{99m}Tc(CO)$_3$(H$_2$O)$_3$]$^+$** [19,20]
    1. In a microwave vial, combine 8 mg $K_2[BH_3CO_2]$, 15 mg Na_2CO_3, 20 mg $Na_2B_4O_7 \cdot 10H_2O$, and 25 mg KOCO[CH(OH)]$_2$COONa·4H$_2$O. Purge the vial for 10 min with argon gas.
    2. Add 4 mL of $^{99m}TcO_4^-$ (~1,100 MBq, ~30 mCi) in 0.9% saline to the vial.
    3. Heat the reaction in a microwave for 3.5 min at 110 °C after 10 s of stirring to ensure thorough mixing of reagents.
    4. Adjust the pH of the solution to 3.5-4 using ~400 µL of 1 M HCl. Verify using pH paper.

2. **Radiolabeling of Tetrazine ligands 1-5**
    1. Dissolve 2 mg of each ligand (compounds **1-5**) in 250 µL MeOH[21].
    2. Add 250 µL of [^{99m}Tc(CO)$_3$(H$_2$O)$_3$]$^+$ (~74 MBq, ~2 mCi) to each solution.
    3. Heat the reaction mixture using a microwave for 20 min at 60 °C.
    NOTE: This step was identical for all 5 tetrazines.
    4. For compounds **2- 5**, evaporate the solvent and re-dissolve the resulting products in 1 mL of 1:1 v/v DCM:TFA.
    5. Heat the dissolved reaction products (**2-5**) at 60 °C in a microwave for 6 min (**2-4**) or 10 min (**5**).
    6. After cooling to room temperature, evaporate the solvent using an evaporator (36 °C, 8 mbar, 3 min, 6,000 rpm) and dissolve the dried compound in 1:1 ACN:H$_2$O or 1:1 MeOH:H$_2$O, prior to HPLC purification.
    7. Purify the ^{99m}Tc-labeled compounds (**1-5**), including separating the labeled product from unlabeled tetrazine ligand, using HPLC (C$_{18}$ reversed-phase). Typically, use an elution gradient of 30:70 ACN:H$_2$O (both with 0.1% TFA) to 40:60 ACN:H$_2$O over 20 min (18 min) and a C$_{18}$ analytical 4.6 x 100 mm column. Use both UV (254 nm) and gamma detection.
        1. Take a small sample of each labeled product and compare its HPLC retention time to that of a co-injected, non-radioactive, Re-labeled standard (0.125 mg in 20% methanol-H$_2$O). The Re-labeled standard is identified in the UV HPLC trace, and will elute at the same time as the ^{99m}Tc-labeled compound in the γ-HPLC trace. This co-injection shows peaks at comparable retention times, confirming the identity of the ^{99m}Tc-labeled compound.
    8. Evaporate the solvent from HPLC fractions using an evaporator (36 °C, 8 mbar, 3 min, 6,000 rpm).
    9. Formulate the purified compound at a concentration of 7.4 kBq/µL in PBS, containing 0.5% BSA and 0.01% Tween-80.
    10. To ensure the labelled compounds are stable, perform an *in vitro* stability study. Incubate the formulated compound at 37 °C for 1, 4 and 6 h, injecting a small amount (3.7 MBq) of the mixture on the HPLC at each time point to assess stability.

2. Pre-targeted Bio-distribution Studies

1. **Preparation of animals**
    1. Using 7-9 week old, female Balb/c mice (n=3), administer TCO-BP formulated in saline (20 mg/kg) (5 µg/µL), via tail-vein injection.

   2. Place mouse in physical restraint device, and identify the veins located on the lateral surfaces of the tail and wipe with an alcohol swab. At approximately 2 cm from the end of the tail, insert a 30-gauge needle at a shallow angle, parallel to the vein. Slowly depress the plunger to inject, remove needle and apply clean gauze sponge at injection site with slight pressure until bleeding stops.
   3. At 1 h post injection of TCO-BP, administer ~0.74 MBq (20 µCi) of ^{99m}Tc-tetrazine formulated in 100 µL of 0.5% BSA, 0.01% Tween-80 in PBS, via tail-vein injection.

2. **Bio-distribution studies**
   1. At the desired time point (t = 6 h), anaesthetize the mice using 3% isoflurane and 2% oxygen gas mixture. Demonstrate a toe pinch withdrawal on the anesthetized mouse to ensure they are under surgical plane of anesthesia.
   2. Collect blood (1 mL) via cardiac puncture using a syringe pre-treated with heparin. Place mouse on its back with nose in the nose cone for continued anesthesia and locate the xiphoid process on the animal.
      1. Insert a 25 G needle, slightly to the left of the animal's midline under the xiphoid process, at a 20° angle. Fully insert the needle, and slowly pull back on the plunger to see blood in the needle hub if the heart was punctured. Slightly readjust the needle while holding the plunger if necessary, to puncture the heart. Slowly draw blood into the syringe.
   3. Euthanize the animal by cervical dislocation, while under anesthesia.
   4. Place each animal in a plastic bag and use a dose calibrator (^{99m}Tc setting) to measure the whole body activity level.
   5. Collect the following tissues and fluids in pre-weighed counting tubes: blood, bone (knee and shoulder), gall bladder, kidneys, liver, stomach (with contents), small intestines (with contents), large intestines and caecum (with contents), thyroid and trachea, urinary bladder with urine, and tail.
   6. Rinse appropriate tissues (excluding blood, gall bladder, and urinary bladder) in PBS to remove blood and blot dry before placing the tissues in appropriate counting tubes.
   7. Place animal carcass in a plastic bag and measure residual whole body activity using a dose calibrator.
   8. Weigh each tube containing a tissue sample. Subtract initial weight of the tube to obtain mass of the tissue.
   9. Use a dose calibrator (^{99m}Tc setting) to measure the amount of activity in a test sample (100 µL) at the time of injection for each mouse. NOTE: This test sample is equal to the injection volume, thus giving the activity count at the time of injection.
   10. At the time of tissue measurement, aliquot 5 µL of the test sample used previously. Use a multi-detector gamma counter (^{99m}Tc setting) and count to obtain the count per minute (CPM) for the 5 µL test sample.
   11. Use the two values obtained in 2.2.9 and 2.2.10 to calculate the activity and CPM relationship using equation *1* to obtain a conversion factor (CPM µCi^{-1}).

$$(1)\ \frac{\textit{Standard CPM} * \left(\frac{\textit{dose cal volume}}{\textit{gamma counter volume}}\right)}{\mu\text{Ci in Standard at time of injection}}$$

   12. Use the gamma counter to measure the amount of radioactivity in each tissue or fluid sample.
   13. Use equation 1 to calculate the amount of activity in each tissue or fluid at the time of measurement relative to the total injected dose. This value is then normalized by organ weight and reported as percent injected dose per gram (*i.e.,* %ID/g) of tissue.
   14. Follow steps 2.1.2 to 2.2.13 to conduct a negative control experiment using the ^{99m}Tc-labeled tetrazine ligands in the absence of TCO-BP. Sacrifice mice (n = 3) at 0.5, 1, 4 and 6 h post injection and obtain tissue or fluid as described above.

Representative Results

The ligands were synthesized using different linkers and chelators via a simple reductive amination strategy (**Figure 2**), followed by coupling of the product to a commercially available tetrazine[22,23]. Radiolabeling was performed using the same method for all compounds and was highly reproducible. The process was optimized by varying the pH, amount of ligand, reaction time and temperature whereupon the ^{99m}Tc-radiolabeled compounds **1-5** were obtained in moderate to high radiochemical yield: 83% (**1**), 45% (**2**), 31% (**3**), 42% (**4**), and 54% (**5**). Following HPLC purification from unreacted ligand and evaporation using an evaporator, the compounds were formulated in PBS containing 0.5% BSA and 0.01% Tween80 prior to injection. The specific activity of the purified ^{99m}Tc-labeled tetrazine was ~1.48 MBq/µg. Studies were conducted to assess the stability of the ^{99m}Tc-labeled tetrazine ligands prior to *in vivo* studies. The stability was monitored by HPLC at 1, 4 and 6 h with no visible degradation over 6 h (R_t= 14 min), as seen in **Figure 3** for compound **4** as an example.

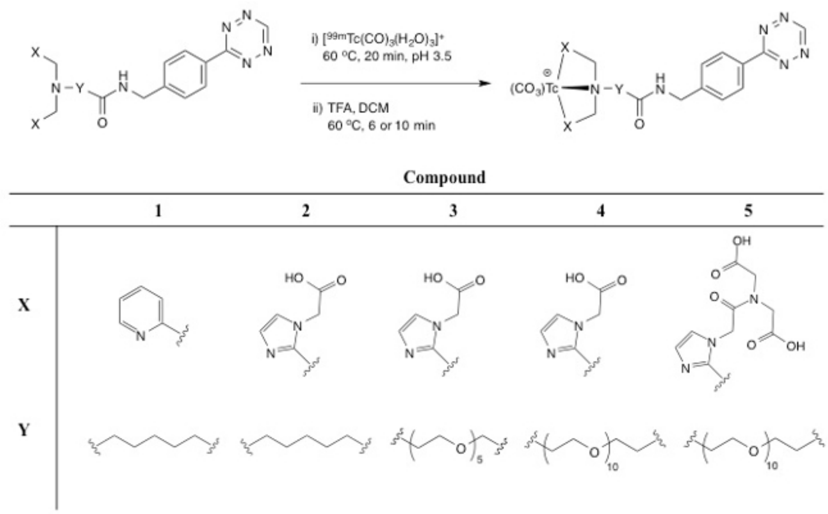

Figure 2: Compounds 1-5 were produced using different linkers (Y) and chelators (X) as shown (bottom). All compounds were radiolabeled with $[^{99m}Tc(CO)_3(H_2O)_3]^+$ using the same reaction conditions (top), with the exception of **1**, **which did not require step (ii)**.

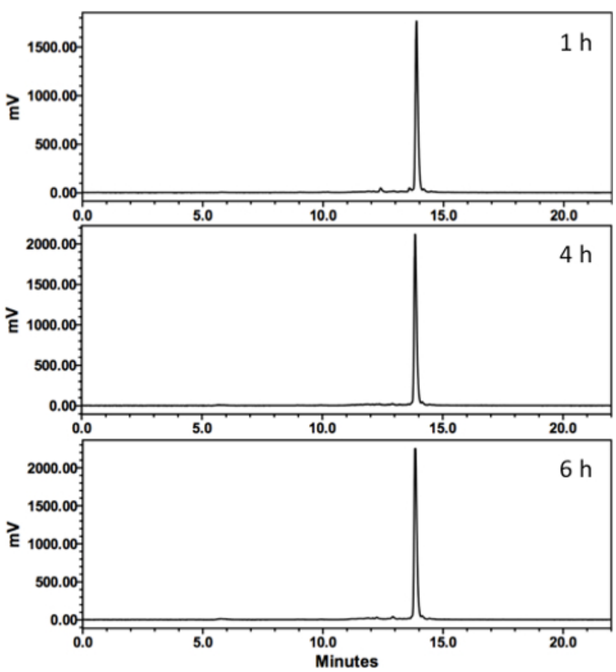

Figure 3: Stability test results using compound 4. γ-HPLC traces of 4 incubated in PBS at 37 °C for 1, 4 and 6 h.

For the *in vivo* testing, healthy Balb/c mice were used. Briefly, for each compound, groups of mice (n = 3) were injected with TCO-BP (100 µL, 20 mg/kg), which was followed by administration of the ^{99m}Tc-labeled compounds 1 h later. At 6 h post-injection of the ^{99m}Tc complexes, the animals were sacrificed and the activity concentrations in various tissues and fluids determined. The resulting data is reported as percent injected dose per gram tissue (%ID/g) and is shown in **Figure 4**. Representative ratios of bone (knee or shoulder) to blood for each of the five ^{99m}Tc-labeled Tz compounds are shown on **Table 1**. These data indicate clearly that compound **3** provided optimal targeting combined with clearance from blood, and that there was substantial variation among the ^{99m}Tc-labeled compounds in regard to off-target tissue localization. A negative control study using CD1 mice (n = 3) was conducted, where mice were injected with ^{99m}Tc-tetrazine ligands in the absence of TCO-BP. Mice were sacrificed at 0.5, 1, 4 and 6 h and %ID/g was determined for all tissues and fluids. For all compounds tested, where data for compound **2** is presented in **Figure 5**, no significant uptake was seen in bone or other tissues (heart, lungs, spleen, skeletal muscle) not shown in **Figure 4**.

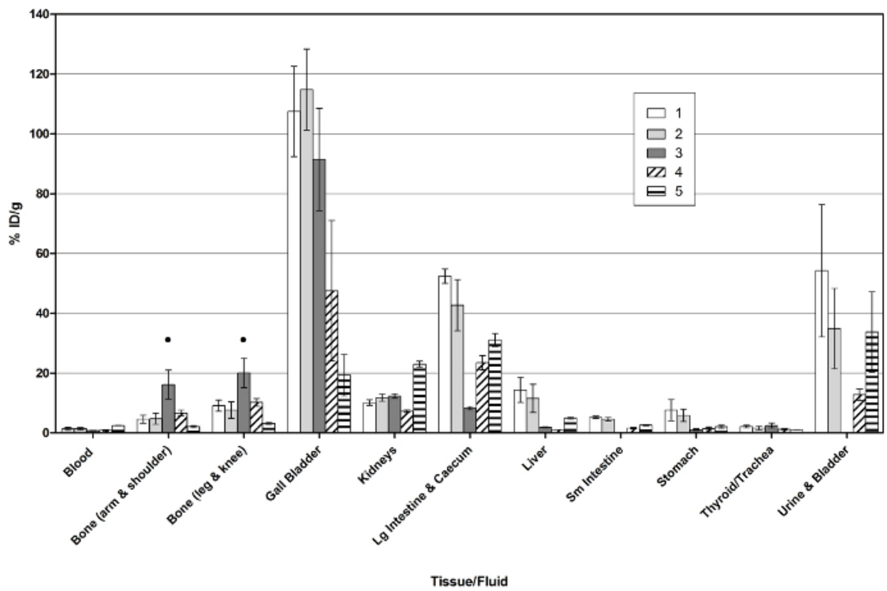

Figure 4. Bio-distribution results for ^{99m}Tc-labeled tetrazine derivatives **1-5** (bars indicated). Data shown were obtained from selected tissues and fluids taken 6 h post injection of the radiolabeled derivatives, and activity was normalized to tissue or fluid weight, as mean percent injected dose per gram of tissue or fluid (%ID/g) ± SEM. Bone targets are indicated by •. NOTE: All remaining tissues not shown had mean %ID/g that was less than 1%. Please click here to view a larger version of this figure.

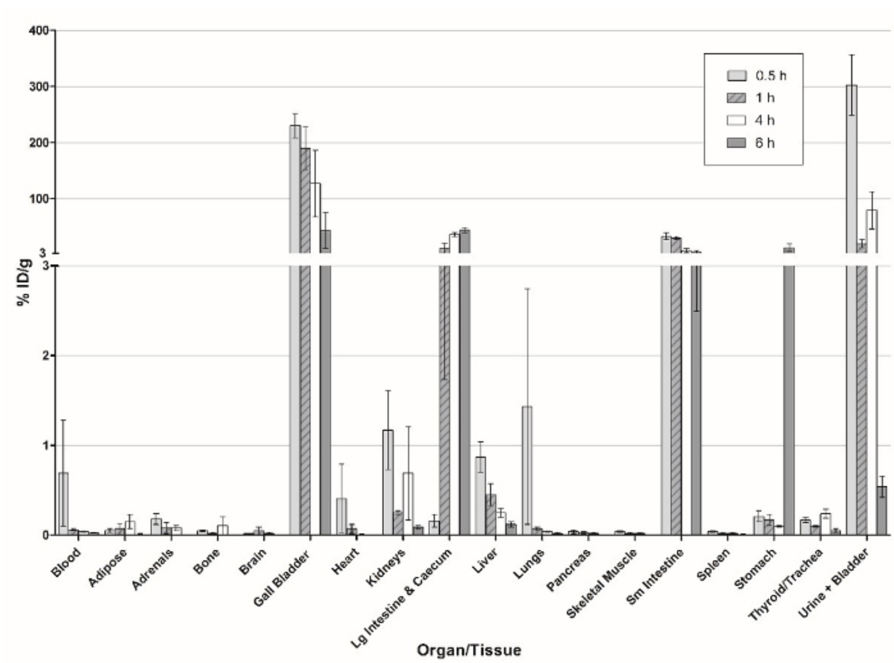

Figure 5. Bio-distribution results for control study using ^{99m}Tc-labeled tetrazine (**2**) without prior injection of TCO-BP. Data shown were obtained from selected tissues and fluids taken from 3 mice at 0.5, 1, 4, and 6 h post injection of **2**. Activity was normalized to tissue or fluid weight, as mean percent injected dose per gram of tissue or fluid (%ID/g) ± SEM. Please click here to view a larger version of this figure.

	Compound				
Ratio	1	2	3	4	5
Shoulder: Blood	3.5 : 1	3.5 : 1	21 : 1	7.8 : 1	0.8 : 1
Knee: Blood	6.9 : 1	5.6 : 1	26 : 1	12 : 1	1.3 : 1

Table 1. Bone tissue: blood ratios determined from bio-distribution studies.

Discussion

A collection of tetrazine-linked tridentate chelates of varying polarities was prepared, and the utility of their ^{99m}Tc complexes in the IEDDA reaction with a TCO derivative *in vivo* was assessed. An effective and reproducible ^{99m}Tc labeling method was developed for five tetrazine-chelates, where the ligand concentration was 10^{-3} M. The labeling step was followed by deprotection of *t*-butyl groups (for compounds **2-5**). The high concentration of ligand was used to improve the radiochemical yield and reduce reaction times which minimized degradation of the tetrazine[21]. The product was isolated and separated from unlabeled ligand and any radiochemical impurities by HPLC, resulting in radiochemical yields ranging from 31-83%, with all having >99% radiochemical purity and a high specific activity of ~1.48 MBq/µg. All compounds were shown to be stable in PBS containing 0.5% BSA and 0.01% Tween80 for up to 6 h (**Figure 3**).

Bisphosphonate compounds, like TCO-BP, localize to regions of active bone metabolism or injury, which include knee and shoulder joints in mice. TCO-BP therefore provides a simple means to assess the effectiveness of new radiolabeled tetrazines to deliver isotopes *in vivo*. Evaluation of the bio-distribution of all five ^{99m}Tc-tetrazines showed uptake in knee and shoulder joints 6 h post injection, demonstrating successful pre-targeting to bone *in vivo* (**Figure 4**). Previous studies confirmed that radiolabeled TCO-BP accumulates at the bone[18], whereas the ^{99m}Tc-tetrazine construct (**2**) given alone does not (**Figure 5**). This allows one to conclude that bone uptake was due to the IEDDA reaction.

The more lipophilic constructs **1** and **2** had similar distribution data including high uptake in the knee (9.1 ± 1.9 (**1**); 7.6 ± 2.7 (**2**)) and the shoulder (4.6 ± 1.4 (**1**); 4.8 ± 1.9 (**2**)). High radioactivity concentrations were also seen in the gall bladder, liver and intestines, which is consistent with the distribution of the lipophilic ^{99m}Tc-tetrazine compound **2** in the absence of TCO-BP (**Figure 5**). Other non-target tissues and organs such as the skeletal muscle and spleen did not show any significant uptake (<1%) when bio-distribution studies were performed on the ^{99m}Tc-tetrazines in the absence of the TCO-BP (**Figure 5**), so these organs were not taken for the pre-targeting experiments. Additionally, bio-distribution experiments with the ^{99m}Tc-tetrazines alone revealed good clearance from non-target tissues at 6 h post injection. Consequently, this time point, which is within one half-life of the isotope, was selected as the time point for comparing the different radiolabeled tetrazine ligands.

The more polar ^{99m}Tc-tetrazine compound **3** bearing a PEG_5 linker showed very high knee and shoulder uptake (16.2 ± 4.8 and 20.7 ± 4.9 respectively). There was also lower activity observed in the liver and intestines. The corresponding PEG_{10} derivative also showed binding to the bone and reduced uptake in the liver compared to compounds **1** and **2.** The most polar derivative **5**, showed lower bone binding than all other constructs which is likely due to its rapid clearance.

The high bone uptake and bone:blood ratios (**Table 1**) particularly for compounds **3** and **4** demonstrate that pre-targeting and the IEDDA reaction can be used to localize ^{99m}Tc-labeled compounds *in vivo*. The methods reported here can be used to evaluate any radiolabeled tetrazine including next generation of Tc(I)-tetrazine ligands. It should be noted that for the class of ligands that were used in this study, the structures can be readily varied by changing the nature of the donor groups and linkers between the metal complex and the tetrazine, without significantly altering the ligand synthesis method[21]. Once a lead molecule is identified, an instant kit method, which will likely include solid phase purification methods, can be developed to support clinical translation.

The Tc(I) complexes reported here create the opportunity to prepare new ^{99m}Tc radiopharmaceuticals using a wide array of different TCO-derived targeting molecules including antibodies. Antibodies, despite their excellent targeting properties prior to the creation of technetium labeled tetrazines, would not typically be used with ^{99m}Tc because of their slow clearance (days), which is much longer than the half-life of the isotope (~6 h). An additional application of the chemistry reported here is that the same class of ligands can be prepared with the beta emitting radionuclides ^{186}Re and ^{188}Re. The isostructural Re(I) analogues of the Tc(I) agents when combined with the tumor seeking properties of TCO-BP can be used to treat bone metastases.

Disclosures

The authors declare they have no competing financial interests.

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