**TITLE:**

Preparation and evaluation of *99mTc-labeled tridentate chelates for pre-targeting using bioorthogonal chemistry*

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99mTc, tetrazine, *trans*-cyclooctene, bioorthogonal chemistry, pre-targeting, imaging.

**SHORT ABSTRACT:**

Here, we describe a protocol for radiolabeling and *in vivo* testing of tridentate 99mTc(I) chelate-tetrazine derivatives for pre-targeting and bioorthogonal chemistry.

**LONG 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 99mTc-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 99mTc 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 99mTc-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.

**INTRODUCTION:**

99mTc remains the dominant radioisotope used in diagnostic nuclear medicine, with over 50 million imaging procedures conducted per year worldwide1–3. The majority of 99mTc agents used clinically are perfusion type radiopharmaceuticals. There are a limited number of actively targeted compounds in which 99mTc is directed to bind a specific biomarker through ligation to a targeting construct. The creation of targeted 99mTc radiopharmaceuticals is often hindered by the influence of 99mTc-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-imaging4–8. 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 effective6. The IEDDA reaction with these components can exhibit fast kinetics in PBS (k2 ≈ 6000 M-1 s-1) and high selectivity, making it ideal for *in vivo* pre-targeting applications9,10.

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 11C, 18F, 64Cu, 89Zr, and 111In have been reported11–15. In contrast, there is only one report of a 99mTc-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 99mTc(I) labeled tetrazines using a family of ligands which form stable tridentate complexes with a [99mTc(CO)3]+ core.

***[please place Figure 1 here]***

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 99mTc-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 used17. 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 99mTc.**

***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.

**1.1)** **Synthesis of [99mTc(CO)3(H2O)3]+** 19,20

1.1.1) In a microwave vial, combine 8 mg K2[BH3CO2], 15 mg Na2CO3, 20 mg Na2B4O7·10H2O, and 25 mg KOCO[CH(OH)]2COONa·4H2O. Purge the vial for 10 min with argon gas.

1.1.2) Add 4 mL of 99mTcO4- (~ 1100 MBq, ~30 mCi) in 0.9% saline to the vial.

1.1.3) Heat the reaction in a microwave for 3.5 min at 110 °C after 10 s of stirring to ensure thorough mixing of reagents.

1.1.4) Adjust the pH of the solution to 3.5-4 using ~400 μL of 1 M HCl. Verify using pH paper.

**1.2)** **Radiolabeling of Tetrazine ligands** **1-5**

1.2.1) Dissolve 2 mg of each ligand (compounds **1-5**) in 250 μL MeOH21.

1.2.2) Add 250 μL of [99mTc(CO)3(H2O)3]+ (~ 74 MBq, ~ 2 mCi) to each solution.

1.2.3) Heat the reaction mixture using a microwave for 20 min at 60 °C.

NOTE: This step was identical for all 5 tetrazines.

1.2.4) For compounds **2**-**5**, evaporate the solvent and re-dissolve the resulting products in 1 mL of 1:1 v/v DCM:TFA.

1.2.5) Heat the dissolved reaction products (**2-5**) at 60 °C in a microwave for 6 min (**2-4**) or 10 min (**5**).

1.2.6) After cooling to room temperature, evaporate the solvent using an evaporator (36 °C, 8 mbar, 3 min, 6000 rpm) and dissolve the dried compound in 1:1 ACN:H2O or 1:1 MeOH:H2O, prior to HPLC purification.

1.2.7) Purify the 99mTc-labeled compounds (**1-5**), including separating the labeled product from unlabeled tetrazine ligand, using HPLC (C18 reversed-phase). Typically, use an elution gradient of 30:70 ACN:H2O (both with 0.1% TFA) to 40:60 ACN:H2O over 20 min (18 min) and a C18 analytical 4.6 x 100 mm column. Use both UV (254 nm) and gamma detection.

1.2.7.1) 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-H2O). The Re-labeled standard is identified in the UV HPLC trace, and will elute at the same time as the 99mTc-labeled compound in the γ-HPLC trace. This co-injection shows peaks at comparable retention times, confirming the identity of the 99mTc-labeled compound.

1.2.8) Evaporate the solvent from HPLC fractions using an evaporator (36 °C, 8 mbar, 3 min, 6000 rpm).

1.2.9) Formulate the purified compound at a concentration of 7.4 kBq/µL in PBS, containing 0.5% BSA and 0.01% Tween-80.

1.2.10) 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 hours, injecting a small amount (3.7 MBq) of the mixture on the HPLC at each time point to assess stability.

1. **Pre-targeted Bio-distribution Studies**

**2.1) Preparation of animals**

2.1.1) 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.

2.1.2) 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.

2.1.3) At 1 h post injection of TCO-BP, administer ~0.74 MBq (20 μCi) of 99mTc-tetrazine formulated in 100 μL of 0.5% BSA, 0.01% Tween-80 in PBS, via tail-vein injection.

**2.2) Bio-distribution studies**

2.2.1) At the desired time point (t = 6 h), anaesthetize the mice using 3% isoflurane and 2% oxygen gas mixture.

2.2.2) 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.

2.2.2.1) Insert a 25-gauge needle, slightly to the left of the animal’s midline under the xiphoid process, at a 20-degree 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.

2.2.3) Euthanize the animal by cervical dislocation, while under anesthesia.

2.2.4) Place each animal in a plastic bag and use a dose calibrator (99mTc setting) to measure the whole body activity level.

2.2.5) 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.

2.2.6) 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.

2.2.7) Place animal carcass in a plastic bag and measure residual whole body activity using a dose calibrator.

2.2.8) Weigh each tube containing a tissue sample. Subtract initial weight of the tube to obtain mass of the tissue.

2.2.9) Use a dose calibrator (99mTc 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.

2.2.10) At the time of tissue measurement, aliquot 5 µL of the test sample used previously. Use a multi-detector gamma counter (99mTc setting) and count to obtain the count per minute (CPM) for the 5 µL test sample.

2.2.11) 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)*

2.2.12) Use the gamma counter to measure the amount of radioactivity in each tissue or fluid sample.

2.2.13) 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.

2.2.14) Follow steps 2.1.2 to 2.2.13 to conduct a negative control experiment using the 99mTc-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 tetrazine22,23. 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 99mTc-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 99mTc-labeled tetrazine was ~1.48 MBq/µg. Studies were conducted to assess the stability of the 99mTc-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 (Rt= 14 min), as seen in Figure 3 for compound **4** as an example.

***[please place Figure 2 here]***

***[please place Figure 3 here]***

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 99mTc-labeled compounds 1 h later. At 6 h post-injection of the 99mTc 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 99mTc-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 99mTc-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 99mTc-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.

***[please place Figure 4 here]***

***[please place Figure 5 here]***

**Figure 1.** The bioorthogonal IEDDA reaction between tetrazine and *trans*-cyclooctene.

**Figure 2.** Compounds **1-5** were produced using different linkers (Y) and chelators (X) as shown (bottom). All compounds were radiolabeled with [99mTc(CO)3(H2O)3]+ using the same reaction conditions (top), with the exception of **1**,which didnot 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.

**Figure 4.** Bio-distribution results for 99mTc-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%.

**Figure 5.** Bio-distribution results for control study using 99mTc-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.

**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 99mTc complexes in the IEDDA reaction with a TCO derivative *in vivo* was assessed. An effective and reproducible 99mTc 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 tetrazine21. 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 99mTc-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 bone18, whereas the 99mTc-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 99mTc-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 99mTc-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 99mTc-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 99mTc-tetrazine compound **3** bearing a PEG5 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 PEG10 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 99mTc-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 method21. 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 99mTc 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 99mTc 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 186Re and 188Re. 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.

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**DISCLOSURES:**

The authors declare they have no competing financial interests.

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