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A Bone-Seeking *Trans*-Cyclooctene for Pretargeting and Bioorthogonal Chemistry: A Proof of Concept Study Using ^{99m}Tc- and ^{177}Lu-Labeled Tetrazines

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Abstract

A high yield synthesis of a novel, small molecule, bisphosphonate-modified *trans*-cyclooctene (TCO-BP, **2**) that binds to regions of active bone metabolism and captures functionalized tetrazines *in vivo*, via the bioorthogonal inverse electron demand Diels-Alder (IEDDA) cycloaddition, was developed. A ^{99m}Tc-labeled derivative of **2** demonstrated selective localization to shoulder and knee joints in a biodistribution study in normal mice. Compound **2** reacted rapidly with a ^{177}Lu-labeled tetrazine *in vitro*, and pretargeting experiments in mice, using **2** and the ^{177}Lu-labeled tetrazine, yielded high activity concentrations in shoulder and knee joints, with minimal uptake in other tissues. Pretargeting experiments with **2** and a novel ^{99m}Tc-labeled tetrazine also produced high activity concentrations in the knees and shoulders. Critically, both radiolabeled tetrazines showed negligible uptake in the skeleton and joints, when administered in the absence of **2**. Compound **2** can be utilized to target functionalized tetrazines to bone and represents a convenient reagent to test novel tetrazines for use with *in vivo* bioorthogonal pretargeting strategies.

Graphical Abstract

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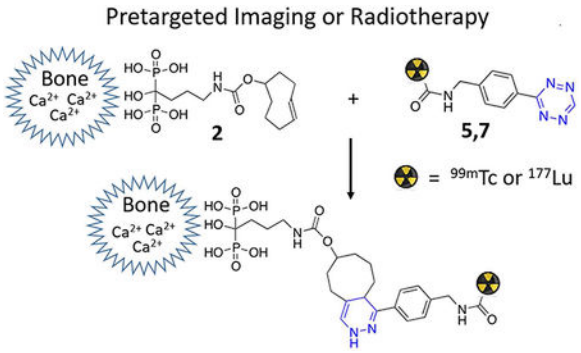
ASSOCIATED CONTENT

Supporting Information: NMR, HRMS and HPLC data; reaction kinetic data; radioTLC data; stability studies; biodistribution data.



INTRODUCTION

Multistep pretargeting based on bioorthogonal chemistry offers an enticing alternative to conventional “active” targeting approaches for nuclear imaging and targeted radiotherapy.[1,2] While a number of different bioorthogonal reactions have been reported, the inverse electron demand Diels-Alder (IEDDA) cycloaddition between tetrazine and *trans*-cyclooctene (TCO) has proven to be highly effective *in vivo* for a wide range of applications.[3–10] To date, the vast majority of investigations focused on pretargeting with IEDDA have centered on the use of radiolabeled tetrazines and antibodies derivatized with TCO,[3–5,7,8,11,12] targeting specific cancer biomarkers. Intriguingly, no bioorthogonal pretargeting strategies aimed at the bone have been developed.

Low molecular weight bisphosphonates (BPs) are used to treat skeletal diseases including osteoporosis, bone metastasis, Paget’s disease, hypercalcemia, and osteoarthritis.[13–17] BPs have also been used as drug delivery vectors,[18] reagents to modify surfaces of bone implant materials for tissue engineering applications,[19] and as radiometal-binding ligands.[20–23] For example, technetium-99m methylene diphosphonate ([^{99m}Tc]-MDP) is widely used to image bone metastases, micro-fractures, and osteomyelitis via single photon emission computed tomography.[24]

A bisphosphonate-modified variant of TCO (TCO-BP) would offer a way to localize diagnostic and therapeutic agents (including but not necessarily limited to medical isotopes) selectively to sites of skeletal disease. A pretargeting strategy using a TCO-BP construct would involve two steps. First, TCO-BP would be administered and rapidly localize to sites of high calcium accretion and active bone metabolism.[15,24] Second, after a pretargeting interval, a labeled tetrazine construct would be injected which would then undergo rapid *in vivo* ligation with TCO-BP residing in the skeleton. Such an approach could have a wide range of clinical applications. For example, in breast cancer patients, pretargeting with TCO-BP and a radiolabeled tetrazine could be used to detect microcalcifications that are often associated with early breast cancer,[25,26] or as a tool to guide biopsies.[27] In addition, a TCO-BP construct would also enable the site-specific immobilization of proteins for bone tissue engineering and the modification of hydroxyapatite-coated medical devices to promote engraftment and biocompatibility and prevent infection.[28–30]

RESULTS AND DISCUSSION

Synthesis of TCO-BP (2).

To test the feasibility and utility of a TCO-BP construct, a model compound was prepared by combining the sodium salt of alendronic acid with commercially available *(E)*-cyclooct-4-enyl-2,5-dioxopyrrolidin-1-yl carbonate (**1**) as detailed in Scheme 1. Unlike typical alendronic acid derivatives, the product **2** could not be precipitated from solution and was consequently isolated by HPLC. Following lyophilization, **2** was obtained as a white solid in 25% yield. The analytical HPLC showed a single peak, as did the ^{31}P NMR (δ 21.8 ppm). The HRMS was also consistent with the mass of the desired product. The product was stable as a solid for at least 3 months when stored in the freezer. The kinetics of the reaction of **2** and a commercially available tetrazine — 4-(1,2,4,5-tetrazin-3-yl)phenyl)methanamine hydrochloride — was evaluated by UV/vis spectroscopy (see Supporting Information). In 1:2 v/v MeOH-saline, the second order rate constant was determined (63.0 ± 0.3 $M^{-1}s^{-1}$), which was similar to that for (E)-cyclooct-4-enol and the same tetrazine as measured in MeOH (81.9 ± 2.3 $M^{-1}s^{-1}$). These results indicate the addition of the BP to TCO had little impact on the rate of the cycloaddition reaction with tetrazines.

Preparation and biodistribution of [^{99m}Tc]-TCO-BP (3).

One convenient approach when employing a pretargeting strategy and attempting to determine the optimal window for administering a radiolabeled tetrazine is to first label the TCO derived molecules and assess its biodistribution.[31] For **2**, we took advantage of the ability of BPs to coordinate ^{99m}Tc where the resulting metal complexes are known to behave like BPs *in vivo* retaining their affinity for sites of active bone metabolism. A method was developed to label **2** with ^{99m}Tc (Scheme 1), in direct analogy to [^{99m}Tc]-MDP, which is used extensively in the clinic to image skeletal diseases, most notably bone metastases.[15,23,32,33] A solution of **2** was combined with $SnCl_2$ and $^{99m}TcO_4^-$. The pH was adjusted to 6.0 by dropwise addition of NaOH, and the reaction was heated to 70 °C for 30 min.[34] For biodistribution studies, **3** was diluted with a solution of **2** to match the concentration that would be used for pretargeting strategies with radiolabeled tetrazines.

The specific structure of Tc-bisphosphonates at the tracer level has not been determined conclusively. However, single crystal X-ray studies at the macroscopic scale with ^{99}Tc showed a polymeric structure.[35] Following the labeling of **2**, radioTLC using acetone and water showed a single product was formed containing no residual pertechnetate or colloidal ^{99m}Tc (see Supporting Information). The stability of the formulated product was initially tested in saline at 37 °C. TLC analysis showed no signs of formation of $^{99m}TcO_4^-$ over 6 h. However, the compound did show significant amounts of colloidal ^{99m}Tc by 6 h (50%). Stability experiments in plasma at 37 °C showed similar results. Given the rapid targeting and clearance of ^{99m}Tc-labeled BPs, there is sufficient stability and amounts of labeled product over 6 h to evaluate the ability of **3** to localize to bone.

Biodistribution studies of [^{99m}Tc]-TCO-BP (**3**) were performed in normal mice at 1, 4, and 6 h post-injection. At all time points, significant activity was observed in bone and joints, but not skeletal muscle (Figure 1). More specifically, the biodistribution results showed rapid

accumulation in the shoulders (8.3 ± 0.2 %ID/g) and knee joints (12.2 ± 0.3 %ID/g) by 1 h. In addition, high activity concentrations were observed in the kidneys (35.5 ± 1.5 %ID/g), most likely a result of the renal clearance of the tracer. Very little uptake was seen in the thyroid and the stomach (see Supporting Information), while modest activity concentrations were observed in the liver (5.0 ± 1.6 %ID/g). The notable uptake in the lung and spleen is likely due to the formation of colloidal ^{99m}Tc, which is consistent with the results observed during the *in vitro* stability studies. With respect to targeting the skeleton and route of excretion, the distribution of **3** is consistent with that reported for other labeled bisphosphonates such as [^{99m}Tc]-MDP.[22,23,36,37] Given the rapid uptake and clearance from the blood, 1 h was selected as the initial time point for administering labeled tetrazines in pretargeting studies.

Evaluation of pretargeting using TCO-BP and an established ^{177}Lu-labeled tetrazine.

Having demonstrated target engagement using [^{99m}Tc]-TCO-BP, we assessed the ability of **2** when bound to bone to capture a radiolabeled tetrazine *in vivo* using a known ligand (Scheme 2). The DOTA-tetrazine (DOTA-Tz) conjugate **4** has been labeled previously with ^{111}In and ^{177}Lu and used with great success to image the biodistribution of TCO-labeled antibodies.[38–40] To prepare the ^{177}Lu-labeled complex, a solution of **4** in NH_4OAc at pH 5.5 was treated with 45 MBq of $^{177}LuCl_3$, and the mixture heated to 60 °C for 5 min. The product, [^{177}Lu]-DOTA-Tz (**5**), was obtained in high (94%) radiochemical yield (see Supporting Information), and was subsequently diluted with saline for biodistribution studies. To assess the reactivity of **5** prior to *in vivo* studies, the product was combined with a solution of **2**, and the reaction mixture monitored by HPLC (see Supporting Information). The analysis showed rapid and complete consumption of **5** and the formation of multiple products associated with the tautomeric and diastereomeric isomers of dihydropyridazine and pyridazine formed following the cycloaddition reaction.[41]

Next, two biodistribution studies were performed in female Balb/c mice. In the first, an active targeting approach was used in which **2** was first combined with **5** to create the [^{177}Lu]-DOTA-Tz-TCO-BP complex prior to injection. In the second, a solution of **2** (approximately 20 mg/kg in saline) was administered i.v., followed 1 h later by **5**. In both experiments, mice were sacrificed at 6 h after injection of the labeled compounds and radioactivity in various tissues and fluids counted. The results indicated near identical biodistribution data for the active targeting and pretargeting strategies (Figure 2). Active targeting produced high activity concentrations in the knee (16.4 ± 2.3 %ID/g) and shoulder (8.3 ± 1.8 %ID/g), similar values to those obtained in the knee (15.5 ± 1.2 %ID/g) and shoulder (9.1 ± 0.8 %ID/g) using the pretargeting approach. Uptake in other major organs — including the kidneys, liver, and gall bladder, and blood — were all less than 2 %ID/g. A higher but modest level of activity (0.8 ± 0.3 %ID/g) was observed in the blood for the pretargeting approach compared to active targeting (0.07 %ID/g). The extremely low blood levels at 1 h for active targeting is evidence that localization of the tetrazine in the pretargeting experiments was due to Tz-TCO coupling in the knee and shoulder rather than in the blood with subsequent migration to the skeleton. It should also be noted that the biodistribution of the [^{177}Lu]-DOTA-Tz (**5**) alone has been reported, and minimal bone uptake was observed.[42] Taken together, these data indicate that **2** effectively targeted regions

of high calcium turnover, which was consistent with the results obtained with **3**, and was accessible for coupling to tetrazines *in vivo*.

Preparation and evaluation of a ^{99m}Tc-labeled tetratrazine.

One of the advantages of TCO-BP is that it can be used to assess novel tetrazine derivatives *in vivo* without having to employ TCO-derived antibodies, which are expensive given the amounts required to perform preclinical pretargeting studies.[43] To demonstrate the utility of **2** in this regard, a new organometallic Tc(I)-tetrazine complex was prepared and evaluated *in vivo*. Tc(I) labeled probes are attractive because they form inert complexes with the appropriate tridentate donors and the labeling precursor, $[Tc(CO)_3(OH_2)_3]^+$ can be obtained in a single step from pertechnetate.[22,23,37,44–47] The low cost, widespread availability, and reduced dose burden with ^{99m}Tc compared to other diagnostic radionuclides makes this an attractive platform for evaluating new targeting vectors. Furthermore, the ability to prepare complementary radiotherapeutic analogues using ^{186}Re or ^{188}Re creates the opportunity to prepare isostructural therapeutic radiopharmaceuticals for treating bone metastases.[4,48]

The Tc(I) binding tetrazine ligand **6** was prepared in high yield in three convenient steps through intermediate compounds **8** and **9** (Scheme 3), using a commercially available tetrazine ((4-tetrazine-3-yl)phenyl)methanamine hydrochloride. The ligand was added to $[^{99m}Tc(CO)_3(OH_2)_3]^+$ at pH 3.5, and the mixture heated for 20 min at 60 °C in a microwave (Scheme 4). After cooling to room temperature the *tert*-butyl ester protecting groups were removed by treatment with 1:1 v/v trifluoroacetic acid (TFA) in dichloromethane (DCM) for 6 min at 60 °C in the microwave. The product [^{99m}Tc]-Tz (**7**) was ultimately isolated by HPLC in 31% radiochemical yield (n = 10) > 99% radiochemical purity (see Supporting Information). The retention time of the Tc complex matched that for the Re standard (**10**). To test the reactivity of **7** with TCO, TCO-BP (0.2 μg) was added to the 4.4 MBq of **7**) in a final volume of 500 μL saline. The resulting conversion was both rapid and complete (Figure 3).

We then performed biodistribution studies using **7** and **2**, in order to compare to the prior biodistribution studies using [^{177}Lu]-DOTA-Tz (**5**). A pretargeting study was performed in which **2** was administered to mice 1 h before [^{99m}Tc]-Tz (**7**), and the results were compared to those of an active targeting study in which [^{99m}Tc]-Tz-TCO-BP complex was formed prior to administration (Figure 4). For pretargeting, biodistribution data at 6 h showed high activity concentrations in the knee (20.07 ± 4.9 %ID/g) and shoulder (16.2 ± 4.8 %ID/g). Minimal uptake was observed in the stomach and thyroid, which is indicative of the stability of Tc(I) complexes. High activity concentrations were also seen in the gall bladder, which is consistent with the biodistribution profile of the parent tetrazine **7**. Critically, the biodistribution of **7** also indicated no uptake in the skeletal bone (see Supporting Information). For active targeting, the data was nearly identical except that higher uptake in the stomach was noted.

BPs ultimately accumulate intracellularly in osteoclasts.[49,50] Consequently, a longer delay prior to the injection of the labeled compound was evaluated to probe the timeframe within which the TCO groups remain accessible on the surface of bone for coupling to a tetrazine.

Compound **7** was administered 12 h following the administration of **2**, using the same concentration employed when the studies were performed with a 1 h delay (Figure 4). At 6 h post injection of **7** (18 h post injection of **2**), significant uptake in the knee (9.6 ± 1.9 %ID/g) and shoulder (6.98 ± 1.9 %ID/g) remained, but these values were over 50% lower than those obtained using a 1 h interval. This suggested that **2** is either increasingly metabolized or internalized by this later time point. In light of the reduced availability of **2**, a significant increase in the activity concentrations in the gall bladder (32.5 ± 7.3 %ID/g) and intestines (9.0 ± 1.9 %ID/g) were also observed.

CONCLUSIONS

In summary, TCO-BP **2** represents an effective ligand for delivering *trans*-cyclooctene to sites of active bone remodeling. As shown in this investigation, this approach can be used for the delivery of both diagnostic (^{99m}Tc) and therapeutic (^{177}Lu) radioisotopes to the bone. Compound **2** has the added advantage that it can be used to rapidly and cost effectively assess novel tetrazine derivatives *in vivo* without having to use antibodies or expensive murine models of disease. As TCO-BP can be synthesized in a single step, the construct can also be readily prepared in large quantities as GMP material, making clinical translation for a range of different applications readily achievable.

EXPERIMENTAL

General Procedures.

Unless otherwise stated, all reagents were used as received from the supplier without further purification. All solvents were purchased from Caledon with the exception of 1,2-dichloroethane (DCE), which was purchased from Sigma-Aldrich (Burlington, ON). Anhydrous solvents were dried using the Pure Solv™ solvent system. Deuterated solvents for NMR samples were purchased from Cambridge Isotope Laboratories (Montreal, PQ). Alendronate sodium trihydrate was purchased from Sigma-Aldrich, and (*E*)-cyclooct-4-enyl-2,5-dioxopyrrolidin-1-yl carbonate was purchased from Click Chemistry Tools (Scottsdale, AZ), and both were used without further purification. Sodium borate, and potassium sodium tartrate ($KNaC_4H_4O_6{\cdot}4H_2O$) were purchased from Anachemia Canada - VWR (Mississauga, ON). Sodium carbonate was purchased from EM Science. Imidazole-2-carboxaldehyde was purchased from AK Scientific (Union City, CA). 1-Ethyl-3-(3-dimethyl-aminopropyl)carbodiimide hydrochloride was obtained from Oakwood Chemical (Estill, SC). Potassium iodide was purchased from BDH Chemicals-VWR. ((4-Tetrazine-3-yl)phenyl)methanamine hydrochloride was purchased from Conju-Probe (San Diego, CA). $NH_2PEG_{10}CH_2CH_2COOH$ was purchased from ChemPeP (Miami, FL). Benzatriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) was purchased from Chem Impex International (Wood Dale, IL). All other compounds were purchased from Sigma Aldrich. Unless otherwise stated, purification was performed by column chromatography using silica gel 60 (particle size 0.04–0.063 mm) purchased from EMD Chemicals – Millipore (Etobicoke, ON) or using a Biotage SP1 system and Biotage SNAP cartridge (KP-Sil 100 g).

Pertechnetate $[^{99m}TcO_4]^-$ was obtained in 0.9% saline from a $^{99}Mo/^{99m}Tc$ generator supplied by Lantheus Medical Imaging. $^{177}LuCl_3$ was purchased from PerkinElmer and used as supplied. ***Caution:*** **These materials are radioactive and should only be used in a properly licensed and equipped facility.** $[^{99m}Tc(CO)_3(OH_2)_3]^+$ was prepared following a literature procedure.[45,46]

Nuclear magnetic resonance spectra (^1H, ^{13}C) were recorded on a Bruker AV600 spectrometer at ambient temperature. All mass spectrometry analyses were provided by the McMaster Regional Centre for Mass Spectrometry. High-resolution mass spectrometry analysis was performed on a Waters/Micromass Q-ToF Global Ultima spectrometer. Microwave reactions were performed using a Biotage Initiator 60 microwave reactor under standard settings, with varying solvents, times and temperatures. Solvents were evaporated under reduced pressure either with a rotary evaporator or using a Biotage V10 system. Radio-TLC was performed using a Bioscan AR-2000 Imaging Scanner on iTLC-SG glass microfiber chromatography paper (SGI0001, Agilent Technologies) plates using distilled water or acetone as the eluent. For each TLC performed, plates were spotted with approximately 2 uL (~3.7 kBq). HPLC of radiolabeled compounds was performed on a Waters 1525 Binary (Midford, MA,) monitored simultaneously with 2998 photo-diode array detector at 220/254 nm or a 2489 photodiode array detector at 214/254 nm and in line radioactivity Bioscan gamma detector with NaI (T1) scintillator using the Empower software package. Analytical and semi-preparative HPLC of all other compounds were performed on an Agilent 1100 series. All synthesized compounds examined and tested were isolated at > 95% purity.

HPLC Method A: Phenomenex Synergi Polar-RP semipreparative column (250×10 mm, 4 μm) operating at a flow rate of 4.0 mL/min was used for purification. The following solvent gradient was employed: (solvent A = H_2O + 0.005% triethylamine (TEA), solvent B = CH_3CN + 0.005% TEA): 0–2 min 2% B, 2–6.5 min 30% B, 6.5–7 min 100% B, 7–10min 100% B, 10–10.5 min 2% B, 10.5–13 min 2% B.

HPLC Method B: Waters X-Bridge C18 column (4.6 × 100 mm, 5 μm,) operating at a flow rate of 1.0 mL/min was used. The following solvent gradient was employed: (solvent A = H_2O + 0.1% TFA, solvent B = CH_3CN + 0.1% TFA): 0–10 min 95–45% A, 10–11 min 45–0% A, 11–15 min 0% A.

HPLC Method C1: A Waters XBridge preparative C-18 column (100 mm × 10 mm × 5 μm) operating at a flow rate of 4 mL/min was used. The following solvent gradient was employed: (solvent A = H_2O + 0.1% TFA, solvent B = CH_3CN + 0.1% TFA): 0–2 min 20% B, 2–19 min 20–80% B, 19–21 min 100% B, 21–22 min 20% B.

HPLC Method C2: Phenomenex C-18 analytical column (250 mm × 4.6 mm × 5 μm) operating at a flow rate of 1.0 mL/min was used. The following solvent gradient was employed: (solvent A = H_2O + 0.1% TFA, solvent B = CH_3CN + 0.1% TFA): 0–2 min 20% B, 2–19 min 20–80% B, 19–21 min 100% B, 21–22 min 20% B.

Chemical Synthesis.

1. Preparation of 2 (TCO-BP): [4-(cyclooct-4-enyloxy carbonylamino)-1-hydroxy-1-phosphono-butyl] phosphonic acid.—(*E*)-cyclooct-4-enyl-2,5-dioxopyrrolidin-1-yl carbonate (29 mg, 0.11 mmol) was dissolved in DMSO (1 mL) and added slowly to a premixed solution of sodium alendronate trihydrate (32 mg, 0.1mmol) and TEA (181 μL, 1.3 mmol) in H_2O (1 mL), and the mixture stirred overnight in the dark. The product was isolated by semi-preparative HPLC using Polar-RP column (method A) and the solvent removed by rotary evaporation. The product was re-dissolved in water and lyophilized producing a white solid. Yield: 10 mg, 25%. ^{1}H NMR (600 MHz, D_2O): δ (ppm) = 5.44 (m, 1 H), 5.31 (m, 1 H), 4.03 (bs, 1 H), 2.94 (q, J = 7 MHz, 6 H), 2.87 (t, J = 6.0 Hz, 2 H), 2.09 (m, 3 H), 1.70 (m, 7 H), 1.53 (m, 2 H), 1.42 (m, 3 H), 1.03 (t, J = 7.2 Hz, 9 H). ^{13}C NMR (150 MHz, D_2O): δ (ppm) = 158.4, 135.7, 133.4, 81.7, 73.7, 46.6, 41.0, 40.4, 38.7, 37.8, 33.7, 32.1, 30.8, 30.6, 24.0, 8.2. ^{31}P{^{1}H} NMR (242 MHz, D_2O): δ (ppm) = 21.77. HRMS (ES^+) *m/z* calcd for $C_{13}H_{24}NO_9P_2$: 400.0926, found: 400.0923. HPLC (method A, UV 220 nm): t_r = 3.9 min.

2. Preparation of 4 (DOTA-Tz): 2,2',20"(10-(2-((4-(1,2,4,5-Tetrazin-3-yl)benzyl)amino)-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid. DOTA-Tz was prepared following literature procedures.[51]

3. Preparation of 8: tert-Butyl 2-(2-formyl-1H-imidazol-1-yl)acetate.— Compound **8** was prepared following a literature procedure.[52] To a solution of imidazole-2-carboxaldehyde (2.02 g, 21.0 mmol) in 10 mL of anhydrous N,N-dimethylformamide (DMF), *tert*-butyl bromoacetate (4.12 g, 21.1 mmol), potassium iodide (120 mg, 0.73 mmol) and N,N-diisopropylethylamine (DIPEA) (5.50 mL, 31.6 mmol) were added. The reaction was allowed to stir for 4 h at 80 °C under argon, whereupon the solvent was removed under reduced pressure. The resulting thick brown oil was extracted with CH_2Cl_2 (3 × 30 mL), and the organic layers combined, extracted with H_2O (3 × 30 mL), and dried over sodium sulfate. The organic layer was evaporated to dryness under reduced pressure to yield a dark brown oil. The desired product was isolated by column chromatography using an SP1 Biotage SNAP 100 g column (1% MeOH/CH_2Cl_2), providing **8** as a yellow oil. Yield: 2.00 g, 9.47 mmol, 45%. TLC R_f =0.26 (1% MeOH/CH_2Cl_2). ^{1}H NMR (600 MHz, $CDCl_3$): δ (ppm) = 9.66 (s, 1H), 7.19 (s, 1H), 7.07 (s, 1H), 4.92 (s, 2H), 1.36 (s, 9H). ^{13}C NMR (150 MHz, $CDCl_3$): δ (ppm) = 181.9, 166.0, 143.4, 131.3, 127.1, 83.1, 49.4, 27.8. HRMS (ES^+) m/z calcd for $C_{10}H_{15}N_2O_3$ [M+H^+] 211.1083, found 211.1077.

4. Preparation of 9: tert-butoxycarbonylmethyl)-1H-imidazol-2-yl]methyl}amino)ethoxy ethoxy}ethoxy)ethoxy]ethoxy}acetic acid.—To a solution of **8** (313 mg, 1.49 mmol) in anhydrous DCE (4.00 mL), NH_2PEG_5COOH (217 mg, 0.734 mmol) was added. The reaction mixture was stirred for 1.5 h at room temperature under argon. Sodium triacetoxyborohydride (465 mg, 2.20 mmol) was then added and the mixture stirred for an additional 12 h. The solvent was evaporated under reduced pressure yielding a clear, pale yellow oil. The desired product was isolated by semi-preparative HPLC (method C1), as a colorless oil following evaporation. Yield: 422 mg, 0.618 mmol, 84%. TLC R_f = 0.43 (10% MeOH/CH_2Cl_2). ^{1}H NMR (600 MHz, $CDCl_3$): δ (ppm) = 8.28 (s, 1H),

6.97 (d, 2H), 6.88 (d, 2H), 4.66 (s, 2H), 4.00 (s, 2H), 3.78 (s, 4H), 3.63 (m, 2H), 3.57 (m, 2H), 3.52 (m, 10H), 3.43 (m, 4H), 2.68 (t, *J* = 6 Hz, 2H), 1.34 (s, 18H). ^{13}C NMR (150 MHz, $CDCl_3$): δ (ppm) = 181.8, 173.7, 166.5, 165.1, 145.0, 131.2, 127.2, 124.9, 121.9, 116.5, 83.1, 70.4, 70.4, 70.3, 69.9, 69.2, 69.2, 53.2, 49.6, 48.0, 27.9, 1.8. HRMS (ES^+) m/z calcd for $C_{32}H_{53}N_5O_{11}$ [$M+H^+$] 684.3823, found 684.3820.

5. Preparation of 6: tert-Butyl {2-[({[1-(tert-butoxycarbonylmethyl)-1H-imidazol-2-yl]methyl}(2-{2-[2-(2-{2-[2-oxo-2-({[p-(1,2,4,5-tetrazin-3-yl)phenyl]methylamino)ethoxy]-ethoxy}ethoxy)ethoxy]ethoxy}ethyl)amino)methyl]-1H-imidazol-1-yl}acetate. In one vial, compound **9** (191 mg, 0.280 mmol), PyBOP (437 mg, 0.84 mmol) and DMF (15 mL) were combined and stirred at room temperature under argon for 15 min. In a separate vial, ((4-tetrazine-3-yl)phenyl)methanamine hydrochloride (76.1 mg, 0.34 mmol), DIPEA (1.30 mL, 7.46 mmol) and DMF (15 mL) were combined and stirred at room temperature under argon for 15 min. The two vials were then combined, and allowed to stir at room temperature under argon for 12 h, whereupon the solvent was removed under reduced pressure. The desired product was isolated by semi-preparative HPLC (method C1), yielding **6** as a bright pink oil. Yield: 50.1 mg, 21%. TLC R_f = 0.26 (10% MeOH/CH_2Cl_2). ^{1}H NMR (600 MHz, $CDCl_3$): δ (ppm) = 10.20 (s, 1H), 8.54 (d, J = 6 Hz, 2H), 7.77 (s, 1H), 7.51 (d, J = 12 Hz, 2H), 7.39 (s, 2H), 7.16 (s, 2H), 4.93 (s, 4H), 4.58 (d, J = 6 Hz, 2H), 4.23 (s, 4H), 4.15 (s, 2H), 3.69 (d, J = 6 Hz, 2H), 3.64 (d, 2H), 3.60 (d, J = 6 Hz, 2H), 3.54 (m, 6H), 3.52 (m, 4H), 3.45 (d, J = 6 Hz, 2H), 2.77 (t, 2H), 1.47 (s, 18H). ^{13}CNMR (150 MHz, $CDCl_3$): δ (ppm) = 171.9, 166.4, 165.1, 158.0, 146.4, 143.4, 131.0, 128.8, 128.7, 123.4, 119.5, 116.8, 114.9, 85.4, 70.8, 70.3, 70.2, 70.0, 69.8, 68.9, 54.0, 49.5, 46.6, 43.0, 28.0, 26.6, 26.5. HRMS (ES^+) m/z calcd for $C_{41}H_{60}N_{10}O_{10}$ [$M+H^+$] 853.4581, found 853.4572.

6. Preparation of 10: tert-Butyl {2-[({[1-(tert-butoxycarbonyl methyl)-1H-imidazol-2-yl]methyl}(2-{2-[2-(2-{2-[2-oxo-2-({[p-(1,2,4,5-tetrazin-3-yl)phenyl]methyl}amino)ethoxy]-ethoxy}ethoxy)ethoxy]ethoxy}ethyl)amino)methyl]-1H-imidazol-1-yl}acetate rhenium tricar-bonyl complex. To a microwave vial, compound **6** (30.5 mg, 0.04 mmol), $Re(CO)_3(H_2O)_3Br$ (20.2 mg, 0.05 mmol) and acetonitrile (ACN) (3 mL) were combined. The vial was sealed and heated in a microwave for 20 min at 60 °C. Solvent was removed under reduced pressure. Next, the mixture was dissolved in a 1:1 solution of DCM:TFA (1 mL) and added to a microwave vial. The vial was sealed and heated in a microwave for 6 min at 60 °C and subsequently the solvent was removed under reduced pressure. The desired product was isolated by semi-preparative HPLC (method C1), yielding **10** as a bright pink oil. Yield: 4.1 mg, 10%. TLC R_f = 0.18 (10% MeOH/CH_2Cl_2). HPLC (method C1) R_t =14 min. HRMS (ES^+) m/z calcd for $C_{36}H_{44}N_{10}O_{13}Re^+$ [$M+H^+$] 1011.2629, found 1011.2660.

Radiosynthesis.

7. Preparation of 3: [^{99m}Tc]-TCO-BP.—100 μL of a solution of TCO-BP **2** (10 mg in 5 mL of 0.2 M NaOH) was combined with 100 μL of a solution of $SnCl_2$ (10 mg in 10 mL of 0.5 M HCl) and 500 μL $^{99m}TcO_4^-$ (307.1 MBq) added. The pH was adjusted to 6.0 by dropwise addition of 0.2 M NaOH and the reaction diluted to total volume of 1.5 mL with saline, vortexed for 30 min and then heated at 70 °C for 30 min. The product was obtained in

97% radiochemical yield and 97% purity, with a specific activity of 1.54 MBq/μg. For biodistribution studies, [^{99m}Tc]-BP-TCO (**3**) was formulated to 740 kBq in 100 μL by dilution with a solution of **2** (10 mg in 2.6 mL). Mice received a maximum dose of **2** of 380 μg per injection (20 mg/kg).

8. Preparation of 5: ^{177}Lu-labeled DOTA-Tz.—To a solution of **4** (100 μg) in 100 μL of 0.2M NH_4OAc (pH 5.5) was added $^{177}LuCl_3$ (45.1 MBq) and the mixture heated to 60 °C for 5 min. The product was obtained in 94% radiochemical yield and purity, with a specific activity of 0.423 MBq/μg of. The solution was diluted with sterile saline and used in reactivity and biodistribution studies. HPLC (method B): R_t = 6.8 min. To prepare the construct for active targeting, 40μL of TCO-BP (**2**, 0.5 mg in 100 μL in saline) was added to a solution of **5** (6.96 MBq in 1.80 mL saline) and the mixture stirred for 10 min. prior to use.

9. Preparation of 7: tert-Butyl {2-[({[1-(tert-butoxycarbonylmethyl)-1H-imidazol-2-yl]methyl}(2-{2-[2-(2-{2-[2-oxo-2-({[p-(1,2,4,5-tetrazin-3-yl)phenyl]methyl}amino)ethoxy]-ethoxy}ethoxyethoxy]ethoxy}ethyl)amino)methyl]-1H-imidazol-1-yl}acetate technetium-99m tricarbonyl complex. To a solution of $[^{99m}Tc(CO)_3(OH_2)_3]^+$ in saline (259 μL, 111 MBq), add 241 μL of a MeOH solution of **6** (10^{-3} M) in MeOH. The vial was sealed and heated in a microwave for 20 min at 60 °C. Upon cooling, the mixture was analyzed for purity by analytical HPLC (method C2, 1 mL/min). After cooling and evaporation of solvent, the tert-butyl ester protecting groups were removed by treatment with 1:1 TFA in DCM and heated in a microwave for 6 min at 60 °C. The solvent was removed and the residue re-suspended in CH_3CN/H_2O (1:1 v/v) and the desired product isolated by HPLC (method C2) in 31% radiochemical yield (n = 10), and a specific activity of 8.88 MBq/μg. To prepare the actively targeted compound, compound **7** (400 μL, 4.44 MBq) was added to TCO-BP **2** (2 μg/μL, 100 μL) in 0.9% saline. The solution was incubated at room temperature for 10 min prior to use.

Biodistribution studies.

Animal studies were approved by the Animal Research Ethics Board at McMaster University in accordance with Canadian Council on Animal Care (CCAC) guidelines. Biodistribution studies were performed using female Balb/c mice (Charles River Laboratories, Kingston, NY) at the indicated time points. The mice were administered the agents via tail vein injection. In the pretargeting studies, a 5 mg/mL solution of compound **2** in saline was administered (20 mg/kg) 1 h prior to the labeled compound. For all studies, at 6 h post-injection of the labeled compounds, animals were anesthetized with 3% isoflurane and euthanized by cervical dislocation. Fluids, bone (knee, shoulder), and select tissues were collected, weighed, and counted in a PerkinElmer Wizard 1470 Automatic Gamma Counter. Decay correction was used to normalize organ activity measurements to time of dose preparation for data calculations. Data is expressed as percent injected dose per gram tissue or fluid (%ID/g) or percent injected dose per organ (%ID/O).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS USED

TCO	*trans*-cyclooctene
IEDDA	inverse electron demand Diels-Alder
BP	bisphosphonate
MDP	methylene diphosphonate
DOTA	1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid
Tz	tetrazine
TEA	trimethylamine
DIPEA, N	N-diisopropylethylamine
ACN	acetonitrile
PyBOP	Benzatriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate
TFA	trifluoroacetic acid
DCM	dichloromethane
DCE	dichloroethane
DMF	dimethylformamide

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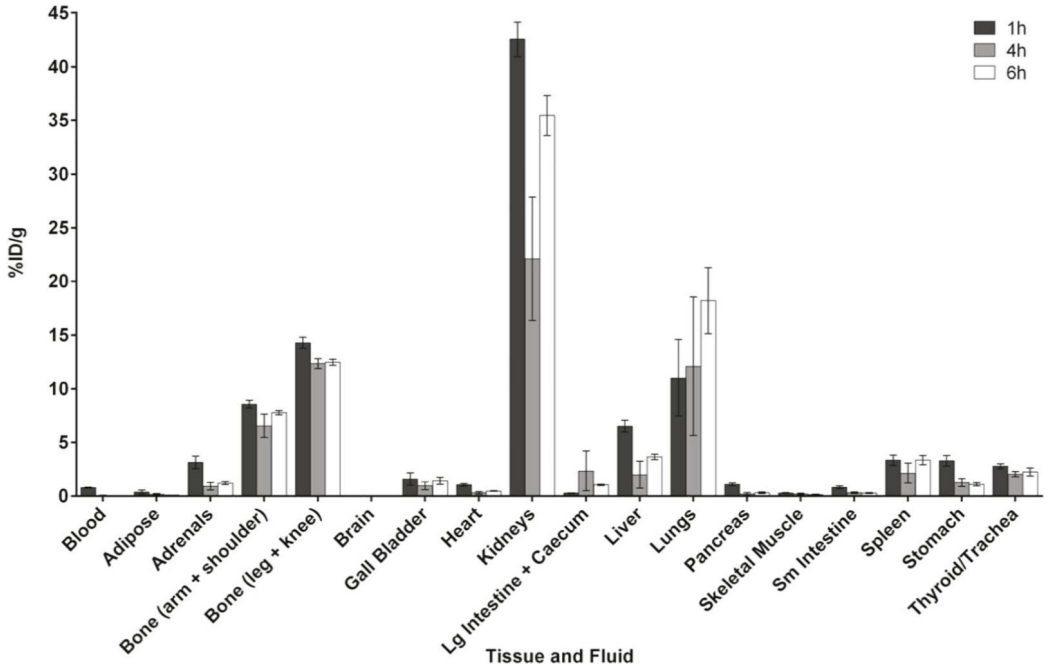


Figure 1.

Biodistribution data for **3** administered to Balb/c mice (n = 3 per time point). Data at the time points indicated are expressed as the mean percent injected dose per gram (%ID/g) ± SEM. Experimental details and numeric values can be found in the Supporting Information.

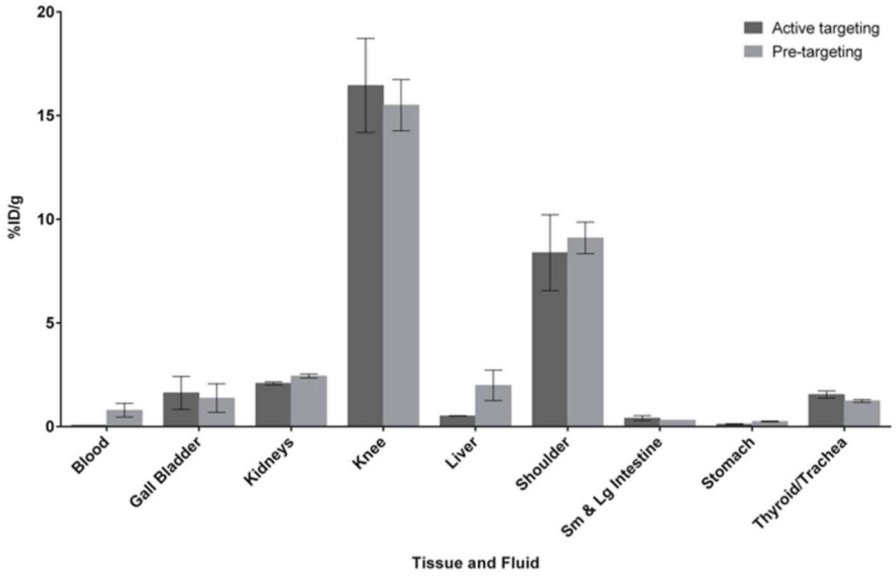


Figure 2.

Biodistribution data for select fluids and tissues for active targeting of **5** combined with **2** (dark bars) prior to administration, and pretargeting (light bars) with 20 mg/kg of **2** administered 1 h prior to **5**. Experiments were performed using Balb/c mice (n = 3 per time point) and tissues collected at 6 h post administration of the labeled compounds. Data are expressed as the mean percent injected dose per gram (%ID/g) ± SEM. Full numeric values of both %ID/g and percent ID per organ are found in the Supporting Information.

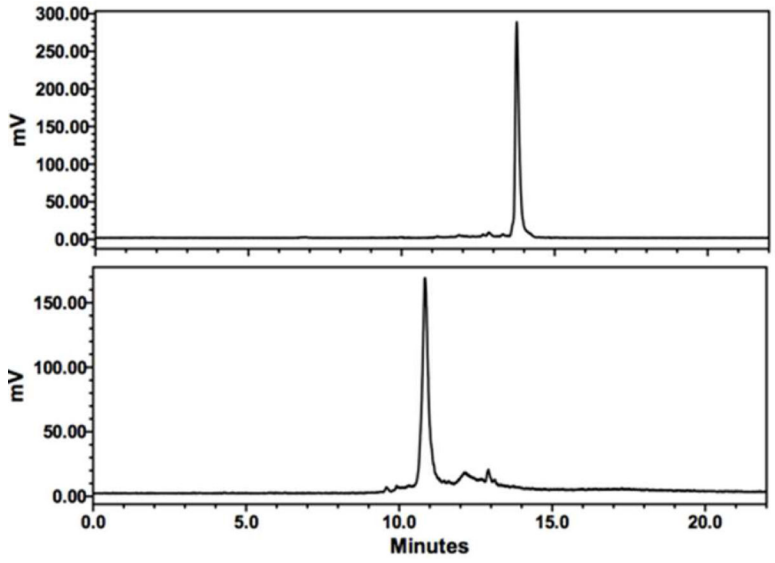


Figure 3.

Gamma HPLC chromatograms of **7** (top) and the reaction mixture following the combination of **2** and **7** (bottom).

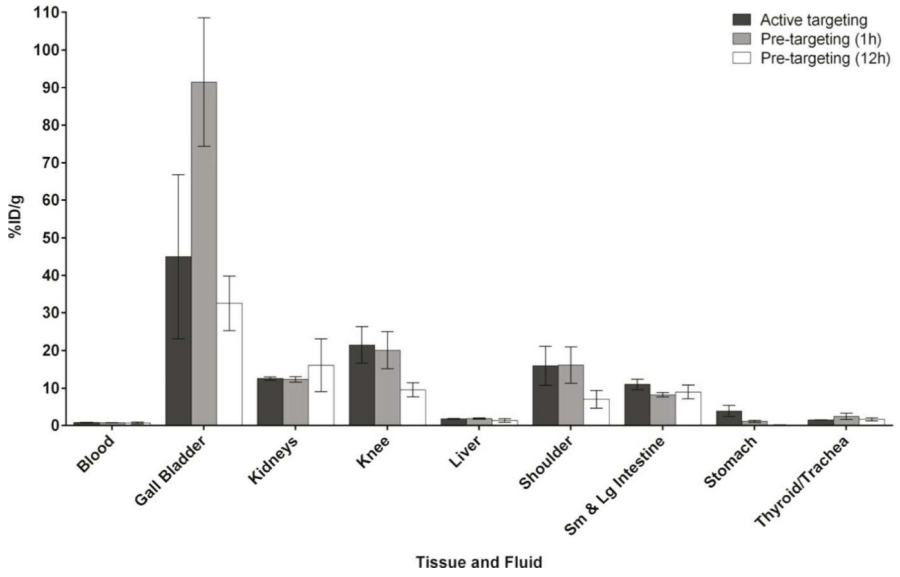
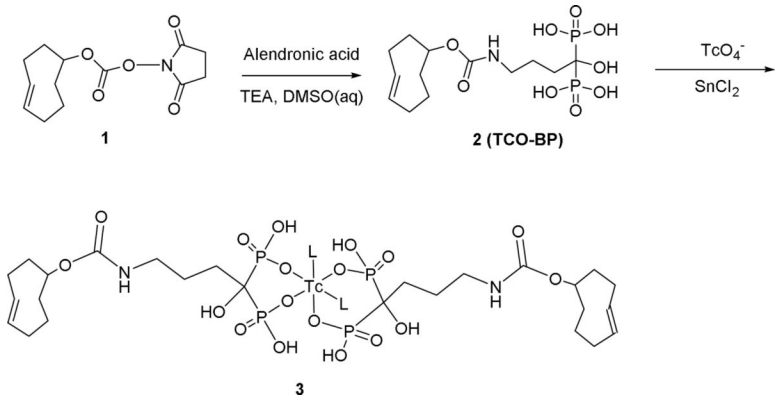


Figure 4.

Biodistribution data for select fluids and tissues for active targeting of **7** combined with **2** prior to administration (dark bars), and pretargeting with 20 mg/kg of **2** administered 1 h (grey bars) and 12 h (white bars) prior to **7**. Experiments were performed using Balb/c mice (n = 3 per time point) and tissues collected at 6 h post administration of the labeled compounds. Data are expressed as the mean percent injected dose per gram (%ID/g) ± SEM. Full numeric values can be found in the Supplementary Information

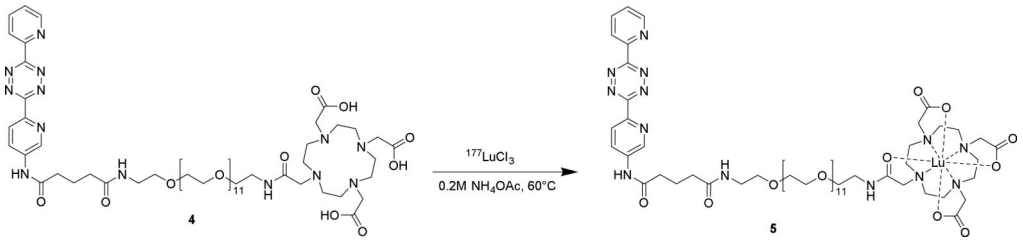


Scheme 1.

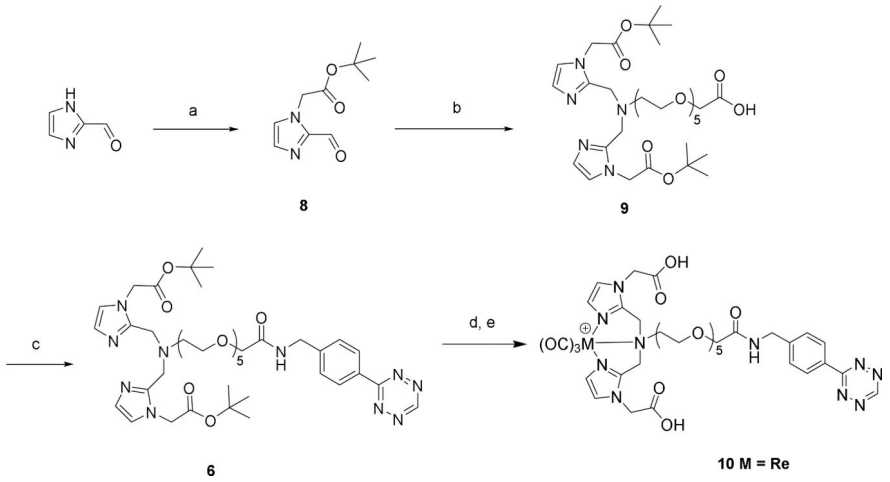
Synthesis of a trans-cyclooctene derivative of alendronic acid (TCO-BP, **2**) and radiolabeling with technetium-99m to generate [^{99m}Tc]-TCO-BP (**3**)

L = OH

Note: The specific structure of Tc-bisphosphonates at the tracer level has not been determined conclusively as it is a mixture of different products.[35] A representative monomeric structure is shown here.



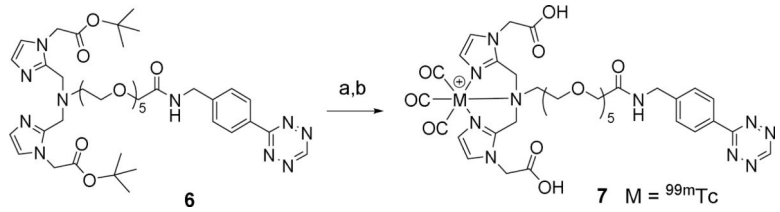
Scheme 2.
^{177}Lu labeling of DOTA-Tz **4**.



Scheme 3.

Synthesis of a tetrazine ligand **6** for binding ^{99m}Tc(I) or Re.

a) *tert*-butyl bromoacetate, DMF, DIPEA, 80 °C, 4 h. b) $NaBH(OAc)_3$, NH_2-PEG_5-CH_2COOH, DCE, 50 °C, 12 h. c) PyBOP, DIPEA, DMF, ((4-tetrazine-3-yl)phenyl)methanamine hydrochloride. d) $Re(CO)_3(OH_2)_3Br$, ACN, 60 °C, 20 min. e) TFA, DCM, 60 °C, 6 min.



Scheme 4.

Synthesis of ^{99m}Tc(I) tetrazine complex **7**.

a) $[^{99m}Tc(CO)_3(OH_2)_3]^+$, 60 °C, 20 min. b) DCM, TFA, 60 °C, 6 min.