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An osteoclast-targeting agent for imaging and therapy of bone metastasis

Wei Liu [a], Asghar Hajibeigi [a], Mai Lin [a], Cynthia L. Rostollan [a], Zoltan Kovacs [b], Orhan K. Öz [a], Xiankai Sun [a,*]

[a] Department of Radiology, University of Texas Southwestern Medical Center at Dallas, 5323 Harry Hines Boulevard, TX 75390, USA
[b] Advanced Imaging Research Center, University of Texas Southwestern Medical Center at Dallas, 5323 Harry Hines Boulevard, TX 75390, USA

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ABSTRACT

A hybrid compound (DO3A-BP) featuring a radiometal bifunctional chelator (1,4,7,10-tetraazacyclotetradecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid, DOTA) and an osteoclast-targeting moiety (bisphosphonate) was designed and synthesized. The ^{111}In-labeled complex of DO3A-BP showed significantly elevated uptake in osteoclasts compared to the undifferentiated adherent bone marrow derived cells. Biodistribution studies revealed a favorable tissue distribution profile in normal mice with high bone uptake and long retention, and low or negligible accumulation in non-target organs.

A variety of cancers preferentially metastasize to the skeleton at their advanced stages. Whole-body scan using ^{99m}Tc-MDP (MDP: methylene diphosphonate) is currently the standard clinical practice for the detection of bone metastases.[1,2] However, due to its low specificity a final diagnosis is often aided by other imaging modalities, such as X-ray radiography, magnetic resonance imaging (MRI), computed tomography (CT), positron emission tomography, and/or bone marrow biopsy.[3,4] Of the clinical methods to treat bone metastases, therapies utilizing bisphosphonates (BPs) and radiopharmaceuticals play critical roles. To date, several BPs have been approved by the FDA[5] and they are commonly used to treat skeletal complications caused by metastases and other bone diseases, including tumor-associated osteolysis,[6,7] hypercalcemia,[8] Paget's disease,[9,10] and osteoporosis.[11] As shown in Figure 1, bisphosphonates are a group of compounds with a chemical structure similar to that of the natural inorganic pyrophosphate (PPi), an endogenous regulator of bone mineralization, but differing in the central atom where BPs have a methylene carbon rather than an oxygen atom in PPi. This structural feature renders BPs resistant to hydrolysis under acidic conditions or by pyrophosphatases. Varieties of BPs could be obtained by tuning the R_2 side chain while leaving the R_1 group intact as either –OH or –H.[12] It has recently become clear that BPs first bind avidly to the bone mineral surface and are subsequently internalized selectively by osteoclasts, where they inhibit the osteoclastic activity and induce apoptosis. In addition, BPs have been found to inhibit tumor cell adhesion to mineralized bone as well as tumor cell invasion and proliferation.[13,14]

The binding of the hydroxyl groups of BPs to Ca^{2+} in hydroxyapitate of bone is responsible for the accumulation of BPs in bone. However, it reduces the coordination sites of ^{99m}Tc-MDP in vivo and subsequently ^{99m}Tc-MDP decomposes into $^{99m}TcO_4$ and BP components. Therefore the bone uptake of ^{99m}Tc-MDP is mainly dependent on the osteoblastic activity, and the purely osteolytic lesion is poorly detectable.[15] DOTA (1,4,7,10-tetraazacyclododecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid) is a commonly used bifunctional chelator for radioimmunodiagnosis and radioimmunotherapy because it is able to form thermodynamically stable and kinetically inert complexes with many divalent or trivalent metal ions.[16–18] To date, few conjugates of DOTA and BPs have been reported but recent research has focused on the improvement of the binding affinity of BP to bone for the applications as delivery vehicles of MRI contrast agents and palliation agents for certain bone diseases.[19,20] Given the high expression of osteoclasts in both osteoblastic and osteolytic lesions, here we report the design and synthesis of an osteo-

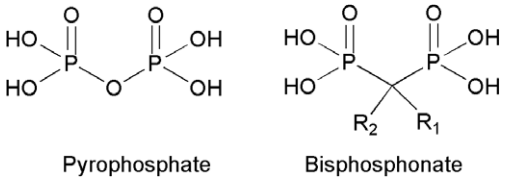

Figure 1. Structures of Pyrophosphate and bisphosphonate.

* Corresponding author. Tel.: +1 214 645 5978; fax: +1 214 645 5885.
E-mail address: Xiankai.Sun@UTSouthwestern.edu (X. Sun).

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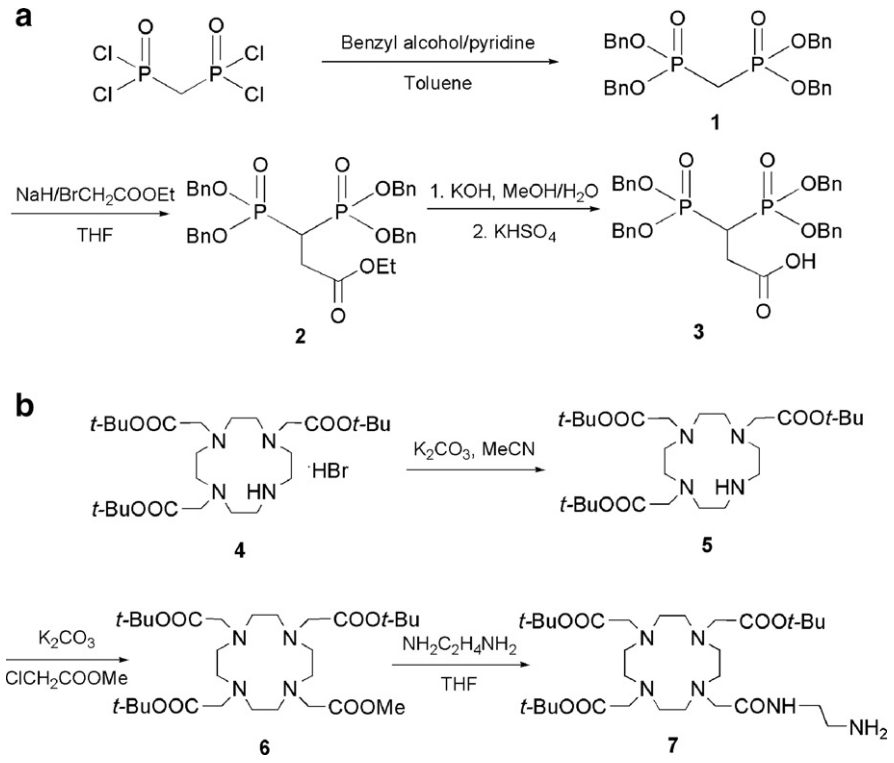
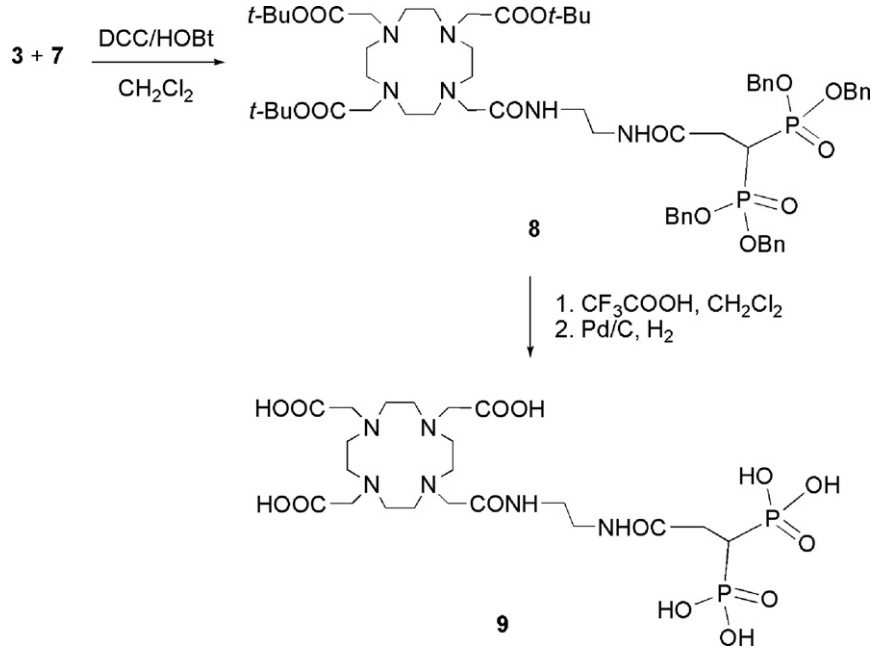


Scheme 1. Synthesis of derivatives of (a) a bisphosphonate and (b) DOTA.

clast-targeting compound by conjugating DOTA to an osteoclast-targeting BP moiety through an ethylenediamine linker, and its in vitro and in vivo evaluation.

A convergent approach was chosen to synthesize the hybrid ligand (DO3A-BP), in which the derivatives of a bisphosphonate (**3**) and DOTA metal chelator (**7**) were synthesized separately as shown in Schemes 1 and 2.

The carboxylate acid substituted bisphosphonate, 3-bis-(dibenzyloxyphosphoryl)propanoic acid (**3**), was synthesized by a literature procedure with an overall yield of 27% (Scheme 1a).[21] The synthesis of **7** was previously reported.[22] However, an alternative route was used in this work (Scheme 2), in which a commercially available intermediate, **5** (1,4,7,10-tetraazacyclododecane-1,4,7-tris(*t*-butyl)acetate: DO3A-*t*-Bu-ester) was used as starting material instead of cyclen and a higher overall yield (77%) was achieved. Alkylation of **5** with methyl chloroacetate afforded **6**, and then **7** was obtained by reacting **6** with excess ethylene diamine. Subsequent conjugation of **3** with **7** via the standard DCC/HOBt



Scheme 2. Synthesis of DO3A-BP (**9**).

procedure (DCC: dicyclo-hexylcarbodiimide; HOBt: 1-hydroxybenzotriazol) (Scheme 2) afforded an orthogonally protected DO3A-BP (**8**) in a yield of 50%. Compound **8** was obtained with high chemical purity via the column chromatography eluted with $CHCl_3$/MeOH (10:1). The ^1H NMR spectrum of **8** showed two well-separated peaks between 8.6 and 9.0 ppm, which can be ascribed to the amide protons on the linkage between the DOTA and BP moieties. A broad peak between 1.8 and 3.5 ppm was observed for the ethylene protons of the macrocycle and it overlapped with the methylene proton signals on the pendent arms. The product, **9** (DO3A-BP), was obtained in nearly quantitative yield after a two-step deprotection, where the *t*-butyl and benzyl protecting groups were removed by trifluoroacetic acid and Pd/C-catalyzed hydrogenation, respectively. Bisphosphonates are typically protected in the form of tetraalkyl bisphosphonate esters and deprotection was conducted by either acid hydrolysis or silylation–dealkylation, however low yields were commonly seen due to the decomposition under the acidic condition of the hydrolysis.[19,23] Our choice of using benzyl rather than alkyl as the BP protecting group enabled us to selectively deprotect **8** in quantitative yield by two separate procedures, which is advantageous to the formation of macrocyclic metal complexes; and provide a UV chromophore for the monitoring of the synthetic procedures.[24]

The radiolabeling of DO3A-BP with ^{111}In was carried out in 0.4 M NH_4OAc buffer, pH 7.5, and monitored by radio-TLC every 4 h via reversed-phase C18 TLC eluted with 10% NH_4OAc/MeOH (v/v: 3:1). Under this TLC condition, free ^{111}In stays at the origin, while the labeled complex migrates with the mobile phase to a certain distance. Although it is well documented that DOTA and its derivatives can be labeled in nearly quantitative radiochemical yields within 2 h under mild conditions,[25,26] such a yield (>95%) could only be obtained after 44 h under similar conditions in the formation of ^{111}In-DO3A-BP.[27] The slow kinetics of DO3A-BP is likely due to the ^{111}In binding competition between the bisphosphonate and the DOTA moieties.[20] An efficient radiolabeling method should be developed for the further application of this agent.

We found that the ability of bisphosphonate to chelate metal ions was dramatically reduced at low pH values due to the protonation of the phosphonate groups, which is consistent with the literature reports.[28,29] Based on this pH-dependent binding manner of BP to metal ions, the ^{111}In-labeled complex was challenged by reducing the pH from 7.5 to 4.0. The low pH was maintained for 3 days, no free ^{111}In was observed. This clearly indicates that the radiometal ion, $^{111}In(III)$, was bound to the DOTA moiety.

The octanol–water partition coefficient ($\log P$) of ^{111}In-DO3A-BP was determined to be -4.05 ± 0.44 ($n = 10$), indicative of the highly hydrophilic nature of the compound. The in vitro serum stability of ^{111}In-DO3A-BP was evaluated out to 48 h by radio-TLC. The concentration of ^{111}In-DO3A-BP was 0.05 mM, while the protein concentration in rat serum was in large excess. The ^{111}In-DO3A-BP complex remained nearly 100% intact within 2 days of incubation with rat serum at 37 °C ($n = 4$). The high in vitro stability of ^{111}In-labeled complex warrants further in vitro and in vivo evaluation.[27]

The osteoclast-targeting property of ^{111}In-DO3A-BP was evaluated on mouse bone marrow derived cells grown for 7 days in the presence or absence of receptor activator for nuclear factor κ B (RANK) and macrophage colony-stimulating factor (M-CSF) ligands according to an established method.[30,31] Osteoclasts are large multinucleate cells formed by the fusion of cells of the monocyte-macrophage lineage in the presence of RANK ligand and M-CSF, which are characterized by high expression of tartrate resistant acid phosphatase (TRAP) and cathepsin K. After incubation for 5–8 days, osteoclasts were formed as seen by cell morphology microscope and TRAP staining (Fig. 2, right). In contrast, bone marrow adherent cells showed negative response to TRAP staining (Fig. 2, left).

For comparison, the uptake in original bone marrow adherent cells (BMCs) and bone marrow macrophages (BMMs) were used as controls. Bone marrow macrophages are considered as the intermediates between BMC and OCs and are formed by treatment with M-CSF only. After a 1-h incubation of ^{111}In-DO3A-BP with the cells, the cells were lysed and the lysates were counted by a γ-counter. The cell uptake of ^{111}In-DO3A-BP was normalized to protein content in each well, which was determined by a commercially available kit. Shown in Figure 3 the osteoclast uptake of ^{111}In-DO3A-BP was about twofold higher than either BMC or BMM, demonstrating its preferential uptake by osteoclasts.[32]

The biodistribution of ^{111}In-DO3A-BP in normal Balb/c mice is presented in Table 1.[33] As anticipated, the ^{111}In-DO3A-BP complex showed high accumulation within 1 h post-injection (pi) in the bone and long residence, which is reflected by only a 20% decrease over 48 h.

In agreement with the reported BP-derivatized compounds,[34] the radiolabeled DO3A-BP showed a relatively high renal uptake, which, however, was rather efficiently cleared. The rapid blood clearance was a result of the high affinity of BPs for bone mineral in vivo.[35,36] The low liver uptake and efficient clearance reflects the high in vivo stability of ^{111}In-DO3A-BP. Impressively, this compound showed no significant uptake in other non-target organs (e.g. lungs, spleen, heart, or brain) and remained intact in the excreted urine within 48 h pi as determined by radio-TLC. The high bone/blood and bone/muscle contrasts further demonstrate the potential of this hybrid compound as a bone-seeking agent.

Taken together, we have synthesized an osteoclast-targeting compound by conjugating DOTA and a BP moiety via an ethylenediamine linker in high overall yields. Our preliminary in vitro and in vivo evaluation results indicate that this compound may find applications in osteoclast-targeted radiotherapy and nuclear imag-

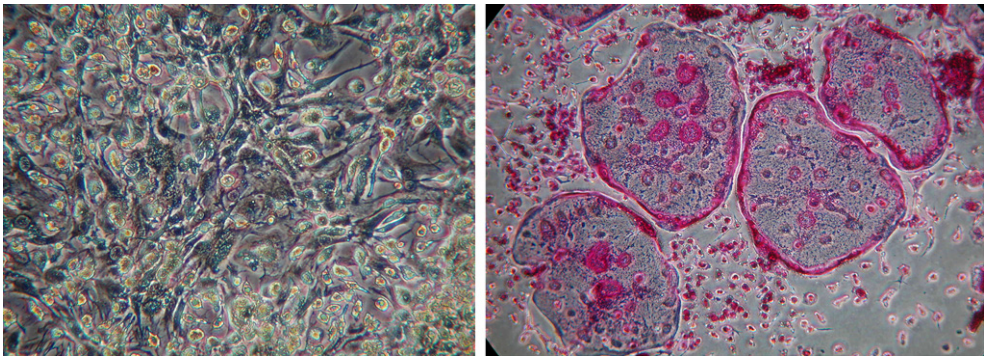


Figure 2. TRAP staining of bone marrow adherent cells (left) and osteoclasts (right).

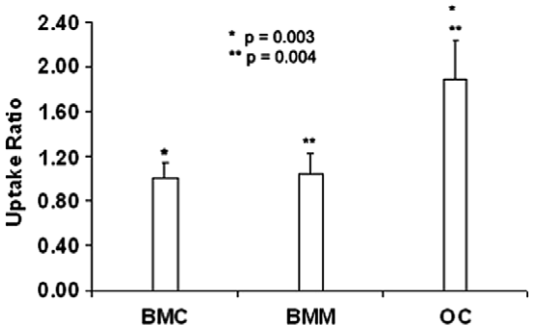


Figure 3. Comparative uptake of ^{111}In-DO3A-BP by osteoclasts (OCs), bone marrow adherent cell (BMC), and bone marrow macrophages (BMMs) at 1 h incubation. Data were obtained from five independent experiments ($n > 6$), and are presented as uptake ratios versus BMC. Gamma counts of the cell lysates were normalized to the protein content of each well for the uptake ratio calculation.

Table 1
Biodistribution of ^{111}In-labeled DO3A-BP in normal Balb/c mice ($n = 4$)

	1 h pi	4 h pi	24 h pi	48 h pi
Blood	0.34 ± 0.08	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Lung	0.52 ± 0.06	0.16 ± 0.04	0.08 ± 0.02	0.08 ± 0.02
Liver	0.41 ± 0.04	0.33 ± 0.05	0.16 ± 0.01	0.14 ± 0.01
Spleen	0.23 ± 0.02	0.15 ± 0.04	0.08 ± 0.01	0.09 ± 0.01
Kidney	4.61 ± 0.42	3.93 ± 1.01	1.16 ± 0.16	0.70 ± 0.09
Muscle	0.49 ± 0.05	0.04 ± 0.01	0.03 ± 0.01	0.05 ± 0.01
Bone	3.39 ± 0.49	2.66 ± 0.81	2.02 ± 0.59	2.60 ± 0.56
B/B	10.12	393.12	644.90	816.49
B/M	6.94	72.91	69.90	49.06

Data are presented as %ID/g ± standard deviation. B/B, bone/blood; B/M, bone/muscle.

ing of bone diseases, which over-expresses osteoclasts relative to osteoblasts or osteoblastic activity, such as multiple myeloma.

Acknowledgments

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24. Chemical synthesis and characterization data.
(a) *General methods.* All chemicals, solvents, and reagents were purchased from Sigma–Aldrich (St. Louis, MO) and used directly without further purification unless otherwise noted. 1,4,7,10-Tetraazacyclododecane-1,4,7-tris-(*t*-butyl acetate) (DO3A-*t*-Bu-ester) was obtained from Macrocylic Inc. (Dallas, TX). Silica gel 60 (70–230 mesh) used for column chromatography was obtained from Sigma–Aldrich. Analytical thin-layer chromatography (TLC) was performed using Merck 60 F254 silica gel (precoated sheets, 0.2 mm thick) (Lawrence, KS) and Whatman MCK 18F reversed-phase plates (Maidstone, Kent, UK). The spectra of ^{1}H NMR, ^{13}C NMR, and ^{31}P NMR were recorded on a Varian 400 or 500 MHz spectrometer; chemical shifts are expressed in ppm relative to TMS (0 ppm), or chloroform (7.26 ppm), and phosphoric acid was used as the external standard. Matrix-assisted laser desorption/ionization (MALDI) mass spectra were acquired on an Applied Biosystems Voyager-6115 mass spectrometer. All reactions were carried out under a nitrogen atmosphere in degassed dried solvents with magnetic stirring. Bulk solvent removal was done by rotary evaporation under reduced pressure, and trace solvent was removed by vacuum pump.
(b) *Compound* **3**. To a suspension of methylene bis(phosphonic dichloride) (5.00 g, 20.0 mmol) in dry toluene (10 mL), a mixture of benzyl alcohol (8.70 mL, 84.0 mmol) and pyridine (6.79 mL, 84.0 mmol) was added dropwise by an addition funnel while the temperature was maintained at 0 °C. The reaction was allowed to reach room temperature and the mixture was stirred for 16 h. The solids formed during the reaction were removed by filtration and washed twice with toluene. The filtrate was washed twice with 2 M NaOH and then once with water. After the removal of bulk solvent, the residue was submitted to column chromatography on silica gel eluting with 100% EtOAc. Evaporation of appropriate fractions afforded **1** as colorless oil. Compound **1** (5.80 g, 10.8 mmol) in THF (50 mL) was slowly added to a suspension of NaH (0.27 g, 11.3 mmol) in THF (100 mL) at 0 °C. After the addition was completed, the reaction mixture was allowed to reach 20 °C and stirred for 30 min. Ethyl bromoacetate (1.91 g, 11.4 mmol) was then added. A white precipitate appeared during the addition. After the mixture was stirred for 48 h, the white solids were removed by filtration and the concentrated residue was submitted to column chromatography on silica gel eluting with EtOAc/Hexane (v/v: 7:3). Evaporation of appropriate fractions gave **2** as colorless oil. A solution of **2** (4.1 g, 6.60 mmol) in methanol (20 mL) was added to 40 mL of a KOH solution (0.46 g, 8.21 mmol) in 50% methanol (aq) at 0 °C. The mixture was stirred at room temperature until **2** disappeared as monitored by TLC. After removal of methanol, the residual was extracted with ethyl ether. The aqueous layer was acidified to pH 2 by adding aqueous $KHSO_4$ (1 M) and then extracted with of $CHCl_3$. The extract was dried over $MgSO_4$ and concentrated in vacuo to give **3** as colorless oil (over all yield 27%): ^{1}H NMR ($CDCl_3$, 500 MHz): δ 2.90 (2H, td, J = 6.5, 16.5 Hz), 3.28 (1H, tt, J = 5.5, 24.0 Hz), 4.95–5.05 (8H, m), 7.20–7.35 (20H, m). MS (MALDI-TOF) 617.6 ($M+Na^+$).
(c) *Compound* **7**. Compound **4** (11.52 g, 19.36 mmol) was dissolved in 100 mL of acetonitrile containing 2 equiv of K_2CO_3. The resulting mixture was stirred at 50 °C for 16 h. After removal of the solids, the filtrate was dried under vacuum. The residue was redissolved in $CHCl_3$ and washed with water thoroughly. The evaporation of $CHCl_3$ afforded **5** in quantitative yield. To 50 mL of **5** (5.26 g, 10.2 mmol) in acetonitrile, K_2CO_3 (3.37 g, 24 mmol) was added followed by methyl chloroacetate (1.14 g, 10.5 mmol) in 5 mL of acetonitrile. The resulting mixture was stirred at 55 °C for 2 days. After removal of the solids and evaporation of the solvent, the residue was redissolved in $CHCl_3$ and washed thoroughly by water. The organic layer was then dried over sodium sulfate. Removal of the solvent under vacuum gave **6** as brown oil, which was used for the next step without purification. A fivefold excess of ethylenediamine was cooled in an ice bath and then mixed with a concentrated solution of **6** in THF. The reaction mixture was stirred at room temperature for 72 h. After evaporation of THF, the excess amine was distilled off at 50 °C under high vacuum. Compound **7** was obtained as a white foam with an overall yield of 77%, which showed high chemical purity as evidenced by: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.45 (27 H, s), 2.52–3.33 (30H, m), 8.76 (1H, br); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 28.45, 42.33, 43.03, 52.22, 52.71, 53.79, 55.15, 56.50, 57.15, 58.42, 81.10, 81.22, 170.84, 170.87, 172.68; MS (MALDI-TOF) 615.8 ($M+H^+$).
Compound **8**.In a round-bottomed flask, compound **7** (200 mg, 0.33 mmol), compound **3** (212 mg, 0.36 mmol), DCC (74 mg, 0.36 mmol), and HOBt (48 mg, 0.36 mmol) were stirred in 20 mL of dry CH_2Cl_2 while cooling in an ice bath. The mixture was then allowed to stand at room temperature with constant stirring for 48 h. The precipitate, dicyclohexylurea, was filtered off, and the filtrate was washed sequentially with aqueous $KHCO_3$ solution 3 times and brine 1 time, and concentrated under reduced pressure to give a white foam. The residue was purified by column chromatography (silica gel 60–230 mesh) using 100% EtOAc to 10:1 $CHCl_3$/MeOH for elution. Compound **8** was obtained

as a sticky oil (192 mg, 50%): ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.36 (27H, multiple), 1.80–3.45 (28H, br), 3.70 (1H, tt, J = 6.0, 24.0 Hz), 4.97 (8H, m), 7.18 (20H, m), 8.71 (1H, br), 8.84 (1H, br); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 28.08, 28.17, 31.61 (t, J = 4 Hz), 33.08 (t, J = 135 Hz), 39.54, 46.45, 48.71 (br), 52.23 (br), 55.69, 55.72, 55.83, 56.26, 68.17, 68.23, 82.00, 128.14, 128.52, 128.63, 136.58, 169.81 (t, J = 8 Hz), 171.67, 172.55; ^{31}P NMR ($CDCl_3$, 162 MHz): δ 25.89 (s); Anal. Calcd for $C_{61}H_{88}N_6O_14P_2 \cdot 6.5H_2O$: C 55.99, H 7.78, N 6.42. Found: C 55.80, H 7.62, N 6.68; MS (MALDI-TOF): 1191.6 (M^+).

(e) *Compound* **9** (DO3A-BP). Compound **8** (40 mg, 0.03 mmol) was dissolved in 3 mL of a mixed solvent of trifluoroacetic acid (TFA) and $CHCl_3$ (v/v: 1:2). The reaction mixture was stirred at room temperature overnight. After removal of bulk solvent under reduced pressure, the residue was dissolved in deionized water and then mixed with 40 mg of Pd/C. The reaction was allowed to proceed in a hydrogenation shaker for 24 h. After removal of the solids, evaporation of water under vacuum gave the target compound (**9**) as a colorless thick oil in nearly quantitative yield: ^{1}H NMR (D_2O, 400 MHz) δ *4.02–2.50* (br); ^{13}C NMR (D_2O, 100 MHz): δ 31.14, 32.35, 33.93, 35.17, 36.41, 38.96, 39.0, 46.0–52.10 (br), 52.10–54.24 (br), 55.0, 174.10; ^{31}P NMR (D_2O, 162 MHz): δ 20.54. MS (MALDI-TOF) 701.8 ($M+K^+$).

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27. Radiochemistry procedures.
(a) *General methods*. All buffers used in radiochemical procedures and in vitro or in vivo experiments were prepared by dissolving salts in Milli-Q water (18 MΩ cm) and then treated with Bio-Rad Chelex 100 resin for removal of trace metal ions. In-111 in 0.05 N HCl was purchased from Trace Life sciences (Denton, Texas). Radio-TLC was performed using a Rita Star Radioisotope TLC Analyzer (Straubenhardt, Germany) on Merck 60 F254 silica gel plates developed by 10% NH_4OAc: MeOH (v/v 3:1) as the mobile phase. Radioactive samples were counted by a Perkin-Elmer γ-counter. Rat serum was purchased from Sigma–Aldrich (St. Louis, MO).
(b) Radiolabeling of DO3A-BP with ^{111}In. To 500 μL of the DO3A-BP ligand solution (0.05 mM) in 0.1 or 0.4 M NH_4OAc buffer (pH 7.5), $^{111}InCl_3$ (ca. 0.5 mCi) in 0.05 N HCl was added. The reaction mixture was incubated at 90 °C in an Eppendorf thermomixer. The formation of ^{111}In-DO3A-BP was monitored by radio-TLC on C18 silica plates eluting with 10% NH_4OAc/MeOH (v/v 3:1).
(c) *Serum stability*. The in vitro serum stability experiment was conducted by adding 10 μL of ^{111}In-DO3A-BP (ca. 50 μCi) to 100 μL of rat serum. The solution was incubated at 37 °C, and sampled for radio-TLC analysis at 1, 4, 24 and 48 h post-addition to rat serum.
(d) Determination of the partition coefficient ($\log P$) of ^{111}In-DO3A-BP was determined by adding 5 μL of the complex to a solution containing 500 μL of Milli-Q water and 500 μL of octanol (obtained from a saturated octanol/water solution) (n = 10). The sample vials were shaken for 1 h at room temperature. From each vial, aliquots of 100 μL each were removed from the octanol phase and the water phase, respectively, and counted separately. The partition coefficient was calculated as the ratio of counts in the octanol fraction to the counts in the water fraction. An average of $\log P$ value was obtained from the 10 samples.
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32. *Osteoclast-targeting evaluation procedures*. M-CSF and soluble RANK were purchased from R&D Systems (Minneapolis, MN); fetal bovine serum (FBS) and heat-inactivated (Δ) FBS were from Atlanta Biologicals (Lawrenceville, GA); the protein assay kit for protein content measurement was from Bio-Rad (Hercules, CA); TRAP staining kit of osteoclastic cells was from Sigma–Aldrich (Diagnostics Acid Phosphatase kit, Procedure No. 387).
(a) Generation of osteoclasts and macrophages from bone marrow cells. Male C57B16 mice were anesthetized and sacrificed by cervical dislocation. The femurs were removed. Both ends of femurs were cut off and bone marrow was centrifuge out from the bone matrix in Hank's Balance Salt Solution (HBSS). Cell clumps were resuspended by pipetting and then filtered through a 70 μm cell strainer. Cells were diluted into 20 mL HBSS and then washed once with HBSS. The cells were resuspended in phenol-free alpha minimal essential medium (α-MEM) containing 10% ΔFBS. After cell counting (trypan blue assay), the cells were seeded into 24-well plates at 5×10^5 cells per well (n = 8). Bone marrow adherent cells were maintained in 1 mL/well of α-MEM containing 10% ΔFBS and 1 × antibiotic at 37 °C in a humidified 5% CO_2 atmosphere. Macrophages were generated in α-MEM containing 10% ΔFBS, 20 ng/mL M-CSF, and 1× antibiotic, while osteoclasts were generated in α-MEM containing 10% ΔFBS, 20 ng/mL M-CSF, 50 ng/mL sRANKL, and 1× antibiotic (1 mL/well) at 37 °C in a humidified 5% CO_2 atmosphere. The media were changed every 2 days. Multi-nucleated giant cells were formed in 5–8 days, which was verified by TRAP staining.
(b) In vitro cell uptake. In a 24-well plate with cell culture as described above, medium was removed and 0.5 mL fresh α-MEM plus 10% FBS containing ^{111}In-DO3A-BP (1 μCi/mL) was added to each well. After 1 h incubation, the medium was removed and the cells were washed with serum free medium (1 mL) twice. The cells were lysed by the addition of 150 μL of Tris-buffered Saline (TBS) containing detergent. The lysates were then transferred to microcentrifuge tubes. Cell uptake of ^{111}In-DO3A-BP was measured by counting the cell lysates in a γ-counter. The protein concentration in each lysate was determined using the Bio-Rad Protein Assay. The assay was repeated 5 times, and all data were pooled for analysis.
33. *Biodistribution*. All animal studies were performed in compliance with guidelines set by the UT Southwestern Institutional Animal Care and Use Committee. Normal male Balb/c mice (18–22 g) were purchased from Harlan (Indianapolis, IN). The solution of ^{111}In-DO3A-BP was diluted with PBS (10 mM) to prepare the injection doses. Each mouse was injected with ca. 5 μCi of radioactivity in 100 μL via the tail vein. The mice were anesthetized prior to sacrifice at each time point (1, 4, 24, and 48 h; n = 4 per time point). Organs of interest were removed, weighed, and counted by a γ-counter. Standards were prepared and counted along with the samples to calculate the percent injected dose per gram (%ID/g) and percent injected dose per organ (%ID/organ). The animals of the last time point groups were housed in metabolic cages (4 mice per cage) to collect urine and feces at 1, 4, 24, and 48 h pi to evaluate the excretion route and stability of the compound.
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