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A comparative study of *trans*- and *cis*-isomers of a bone-seeking agent, DO2A2P

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Abstract

The macrocyclic bone-seeking agent, DO2A2P, bears a cyclen core and two pairs of peripheral phosphonate and carboxylate groups. The geometric disposition of the peripheral functionalities gives rise to a pair of geometric isomers: *cis*-DO2A2P and *trans*-DO2A2P. In order to compare the biological behavior of the isomer pair, *cis*-DO2A2P was synthesized. Both isomers were successfully radiolabeled with ¹⁷⁷Lu, which might potentiate their applications in both radiotherapy and imaging of bone diseases. Through a set of biological assays including the hydroxyapatite binding, *in vitro* stability, and *in vivo* distribution, we demonstrated that the geometric pair of DO2A2P had virtually identical biological properties.

Keywords

Bone-seeking agent; Bisphosphonate; Geometric isomer; Macrocyclics; Lutetium-177

Bisphosphonate analogs have been long used directly (e.g. Pamidronate) or when labeled with therapeutic radioisotopes (e.g. Quadramet[®], ¹⁵³Sm-EDTMP) for palliative treatment of bone metastases, which are common in many types of cancers at advanced stages.¹ In addition, clinical bone scans are mainly performed with ^{99m}Tc-labeled bisphosphonates, such as ^{99m}Tc methylenebisphosphonate (Medronate). Despite the well-established clinical use, directly radiolabeled bisphosphonate complexes suffer several obvious limitations, which include low kinetic stability, suboptimal specific radioactivity, nonspecific accumulation in non-target tissues, and unknown coordination structure.² Therefore, significant efforts have been seen to develop bone seeking agents derivatized from

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bisphosphonates, in which the radiometal is coordinated by a “bifunctional chelator” that oftentimes carries a spacer to optimize the biological behavior.² In this regard, macrocyclic chelators are preferred over their acyclic counterparts because more kinetically stable complexes can result from their rigid and pre-organized structures.^{3–6} Furthermore, macrocyclic ligands bearing a mixed set of phosphonate and carboxylate groups, such as TRITP, DOTP, DOA3P, and *trans*-DO2A2P (Figure 1), have been reported as novel bone targeting agents when labeled with radioisotopes (e.g. ⁶⁸Ga-DOTP, ¹⁵³Sm/¹⁶⁶Ho-TRITP, and ¹⁷⁷Lu-DOTP).² In this class of complexes, both the macrocyclic core and the peripheral phosphonate or carboxylate moieties contribute to the metal binding, while the phosphonate still maintains the desired bone targeting capability. It has been reported that a positive correlation exists between the bone uptake and the number of methylphosphonate pendent arms.^{2, 7} While more phosphonate pendent arms lead to higher bone accumulation, agents with mixed phosphonate and carboxylate groups (e.g. *trans*-DO2A2P) present quicker radiolabeling, higher *in vivo* stability, and relatively lower osmolarity than those bearing a high number of phosphonates (e.g. TRITP, DOTP, and DOA3P).^{7–9}

Unlike TRITP, DOTP, and DOA3P, DO2A2P can present two geometric (*cis/trans*) isomers, which might incur different biological behavior after labeling with radioisotopes.⁷ While the synthesis and biological properties of *trans*-DO2A2P has been well-documented, its *cis*-counterpart, *cis*-DO2A2P (2,2'-(7,10-bis(phosphonomethyl)-1,4,7,10-tetraazacyclododecane-1,4-diyl)diacetic acid), has not been reported. It would be of interest to know whether or not the two geometric isomers behave similarly *in vivo*.

The synthesis of *cis*-DO2A2P was accomplished by a similar approach reported for *trans*-DO2A2P (Scheme 1).¹⁰ First, the 1,4-bis-substituted cyclen, **1**, was synthesized according to a previously reported procedure.¹¹ It was then converted to **2** using triethyl phosphate and paraformaldehyde in a Mannich-type reaction. The intermediate **2** was directly deprotected by refluxing it in hydrochloric acid to afford *cis*-DO2A2P, **3**. The product was further purified by recrystallization from ethanol/water.¹²

Lutetium-177 was chosen for the radiolabeling and biological evaluation of DO2A2P ligands given the fact that ¹⁷⁷Lu (E_{β-max}: 497 KeV; t_{1/2}: 6.73 d) has been increasingly used for targeted therapy and it is a commercially available. In addition to its low-energy β⁻ emission that provides a tissue penetration range effective for the treatment of small tumors (3.0 mm),¹³ ¹⁷⁷Lu's γ energies (113 KeV, 208 KeV) are also suitable for single-photon emission computed tomography (SPECT) imaging, which enables the desirable non-invasive monitoring and radiation dosimetry calculation. The labeling of both *cis*- and *trans*-DO2A2P ligands with ¹⁷⁷Lu was carried out in virtually quantitative yield at 50 °C and 90 °C within 30 min (Table 1).¹⁴ However, when the radiolabeling reactions were conducted at room temperature (r.t.), the isomer pair indeed behaved differently. The *cis*-isomer needed a much longer time (up to 3.5 h) to be completely labeled than the *trans*-isomer (within 1 h).

The hydrophilicity of the radiolabeled complex is one important factor to determine its *in vivo* profile, which can be measured by their partition coefficients (logP) in a biphasic solvent system of *n*-octanol and water.¹⁵ The results indicate both ¹⁷⁷Lu-labeled *cis*- and *trans*-DO2A2P complexes are highly hydrophilic. The *in vitro* stability of both complexes

was evaluated in rat serum by radio-TLC. Both complexes remained virtually intact (> 98%) out to 7 days.¹⁶

The comparative binding of ¹⁷⁷Lu-labeled *cis*- and *trans*-DO2A2P onto hydroxyapatite (HA), the main mineral component of bone, is shown in Figure 2.¹⁷ The adsorption profiles of both ¹⁷⁷Lu-complexes to HA are nearly identical.

In order to compare their *in vivo* bone-seeking profiles, a SPECT/CT imaging study was performed in normal BALB/c mice using both ¹⁷⁷Lu-*cis*-DO2A2P and ¹⁷⁷Lu-*trans*-DO2A2P.¹⁸ As shown in Figure 3, both agents presented almost identical *in vivo* distribution patterns in that they preferentially accumulated in the spine and the bone joints at 1 h post-injection. The quantitative analysis of the SPECT/CT images revealed the bone uptake levels of ¹⁷⁷Lu-*cis*-DO2A2P and ¹⁷⁷Lu-*trans*-DO2A2P were 1.2 ± 0.2 %ID/g and 1.1 ± 0.1 %ID/g at 1 h post-injection, respectively. No significant difference was observed between the two radiotracers. At 24 h post-injection, most of the radioactivity had cleared out of the mouse body.

Taken together, no significant difference was observed for the ¹⁷⁷Lu-labeled geometric isomer pair of DO2A2P in their biological properties assayed in this work.

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12. Chemical synthesis and characterization data: Paraformaldehyde (0.16 g) was added to a mixture of 1,4-DO2A-tBu ester (**1**) (1.00 g, 2.5 mmol) and triethyl phosphate (0.95 g, 5 mmol). The mixture was stirred for 4 days at room temperature. The volatile impurities were removed in vacuum at 60 °C for 1 day to yield clear oil that consisted of the ester intermediate (**2**: di-tert-butyl 2,2'-(7,10-bis((diethoxyphosphoryl)methyl)-1,4,7,10-etraazacyclododecane-1,4-diyl)diacetate) and a small amount of diethyl hydroxymethyl phosphonate. It was dissolved in hydrochloric acid (20%, 30 mL), and the solution was refluxed for 2 days. The hydrochloric acid was removed by rotary evaporation and the residue was redissolved in water, treated with activated carbon (0.5 g), filtered, and evaporated again. The residue was redissolved in a small amount of water (3 mL), and

ethanol (30 mL) was added in small portions. A white amorphous solid precipitated. The solution was decanted, and the residue was redissolved in water (5 mL), and the crystallization of the product was induced by the addition of ethanol (5 mL). The mixture was stirred for 24 h. The white crystalline solid was filtered, washed with water (2×3 mL), and dried to give 0.7 g *cis*-DO2A2P (**3**) (1.47 mmol, yield 59%): ^1H NMR (400 MHz, D_2O , ND_3 , pD: 9–10) δ : 3.72 (4H, s, CH_2COOH), 3.11–3.08 (8H, d br, NCH_2 ring), 2.96–2.93 (8H, m br, NCH_2 ring), 2.72 (4H, d, $J_{\text{H-P}} = 10.0$ Hz, $\text{CH}_2\text{PO}_3\text{H}_2$). ^{13}C NMR (100 MHz, D_2O , ND_3 , pD: 9–10) δ : 177.40 (COOH), 59.13 (NCH_2COOH), 54.12 ($\text{NCH}_2\text{PO}_3\text{H}_2$, $J_{\text{C-P}} = 129.4$ Hz), 53.10 (NCH_2 ring), 53.01 (NCH_2 ring), 52.56 (NCH_2 ring), 52.49 (NCH_2 ring). ^{31}P NMR (161 MHz, D_2O , ND_3 , pD: 9–10) δ : 6.24. m/z (MALDI/TOF): 477.5 (100% $[\text{M} + \text{H}]^+$), calcd m/e , 476.1. Anal.: Calculated for $\text{C}_{14}\text{H}_{30}\text{N}_4\text{O}_{10}\text{P}_2 \times 3.4\text{H}_2\text{O}$; C, 31.28; H, 6.90; N, 10.42. Found, C, 31.35; H, 7.07; N, 10.37.

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14. Radiochemistry procedures: All buffers used in radiochemical procedures and *in vitro* or *in vivo* experiments were prepared by dissolving salts in Milli-Q water (8 M Ω cm) and then treated with Bio-Rad Chelex 100 resin for removal of trace metal ions. Lu-177 in 0.05 N HCl was purchased from University of Missouri Research Reactor. To 100 μL of the DO2A2P ligand solution (5 mM in 0.4 M NH_4OAc buffer, pH 6.5), ^{177}Lu (ca. 37 MBq/1 mCi) in 0.05 N HCl was added. The reaction mixture was incubated at three temperature conditions (r.t., 50 $^\circ\text{C}$, and 90 $^\circ\text{C}$). The formation of ^{177}Lu -labeled DO2A2P was monitored by radio thin layer chromatography (radio-TLC). Radio-TLC was performed using a Rita Star Radioisotope TLC Analyzer (Straubenhardt, Germany) on Whatman TLC plate KC18F reverse phase plate developed by 10% NH_4OAc : MeOH (v/v 1:1) as the mobile phase. Under the TLC condition employed, ^{177}Lu labeled *trans*-DO2A2P and *cis*-DO2A2P migrated with the mobile phase to $R_f = 0.6$ –0.7, while unreacted ^{177}Lu stayed at the original. The labeling yields of both complexes were over 99%.
15. Determination of the partition coefficient (logP): 5 μL of ^{177}Lu -labeled DO2A2P was added to a solution containing 500 μL of Milli-Q water and 500 μL of *n*-octanol (obtained from a saturated octanol/water solution) ($n = 3$). The sample vials were shaken for 1 h at room temperature. From each vial, aliquots of 100 μL each were removed from the *n*-octanol phase and the water phase, respectively, and counted separately by a Perkin-Elmer γ -counter. The partition coefficient was calculated as the ratio of counts in the octanol fraction to the counts in the water fraction. An average of logP value was tried from the 3 samples.
16. Serum stability: The *in vitro* serum stability experiment was conducted by adding 10 μL of ^{177}Lu -labeled DO2A2P (3.7 MBq/0.1 mCi) to 100 μL of rat serum. The solution was incubated at 37 $^\circ\text{C}$, and sampled for radio-TLC analysis up to 7 days post-addition to rat serum.
17. Adsorption study: The ^{177}Lu -labeled *cis*- and *trans*- DO2A2P (370 KBq) was incubated by continuous mixing for 1 h at room temperature with 5, 10, 25, 50, 100, and 150 mg of hydroxyapatite (HA) in 1.5 mL of 0.1 M tris-HCl buffer (pH 7.4), respectively. Immediately after incubation, the mixture was centrifuged at 10,000 rpm to achieve complete sedimentation, and the liquid phase was separated. The solid phase was washed twice with 1.5 mL of 0.1 M tris-HCl buffer (pH 7.4). Washing buffer was pooled with liquid phase. The radioactivity in the liquid and solid phases was measured in a dose calibrator.
18. SPECT/CT imaging procedures: SPECT/CT imaging was performed in a dedicated, multiplexed multi-pinhole small animal SPECT/CT imaging system, NanoSPECT/CT Plus (BioScan). The detection energy window for ^{177}Lu was set at and keV with a 10% difference. The CT projections were recorded at 45 kV and 170 μA of tube voltage and current, respectively. The solution of ^{177}Lu -*cis*-DO2A2P and ^{177}Lu -*trans*-DO2A2P was diluted with saline solution to prepare the injection doses, respectively. Each male BALB/c mouse (18–22 g) was injected intravenously with 20–22 MBq of radioactivity in 0.15 mL. Two imaging sessions started 1 h and 24 h post-injection, respectively ($n = 3$).

Figure 1.

Structures of relevant bisphosphonate (BP) analogs. DO3A-BP: 2,2',2''-(10-(2-((2-(3,3-diphosphonopropanamido)ethyl)amino)-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid; NOTA-HBP: 2,2'-(7-(1-carboxy-4-((4-hydroxy-4,4-diphosphonobutyl)amino)-4-oxobutyl)-1,4,7-triazonane-1,4-diyl)diacetic acid; DOTP: ((1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrayl)tetrakis(methylene)) tetrakis(phosphonic acid); TRITP: ((1,4,7,10-tetraazacyclotridecane-1,4,7,10-tetrayl)tetrakis(methylene)) tetrakis(phosphonic acid); DOA3P: 2-(4,7,10-tris(phosphonomethyl)-1,4,7,10-

tetraazacyclododecan-1-yl)acetic acid; *trans*-DO2A2P: 2,2'-(4,10-bis(phosphonomethyl)-1,4,7,10-tetraazacyclododecane-1,7-diyl)diacetic acid; and *cis*-DO2A2P: 2,2'-(7,10-bis(phosphonomethyl)-1,4,7,10-tetraazacyclododecane-1,4-diyl)diacetic acid).

Figure 2. Comparative hydroxyapatite (HA) adsorption profiles of ^{177}Lu -cis-DO2A2P and ^{177}Lu -trans-DO2A2P versus the HA amount.

Figure 3. Representative SPECT/CT images of ^{177}Lu -*cis*-DO2A2P and ^{177}Lu -*trans*-DO2A2P at 1 h post-injection presented as maximum intensity projection (MIP). *CT images: gray color scale; SPECT images: red color scale (MIP); sagittal, and coronal: nih fire color scale.* Arrows indicate the spine and bone joints.

Scheme 1.

Synthesis of *cis*-DO2A2P. i) triethyl phosphate, paraformaldehyde, 4 days at r.t.; ii) 20% HCl, reflux for 2 days.

Table 1

Radiolabeling efficiency of the *cis*- and *trans*-DO2A2P isomer pair with ^{177}Lu at room temperature (r.t.), 50 °C, and 90 °C.

Time	$^{177}\text{Lu-cis-DO2A2P}$			$^{177}\text{Lu-trans-DO2A2P}$		
	r.t.	50 °C	90 °C	r.t.	50 °C	90 °C
0.5h	53	>99	>99	96	>99	>99
1h	74	>99	>99	>99	>99	>99
2h	91	/	/	>99	/	/
3.5h	>99	/	/	>99	/	/

/ not tested