

Xiankai Sun · Melinda Wuest · Zoltan Kovacs
A. Dean Sherry · Ramunas Motekaitis · Zheng Wang
Arthur E. Martell · Michael J. Welch
Carolyn J. Anderson

In vivo behavior of copper-64-labeled methanephosphonate tetraaza macrocyclic ligands

Received: 13 March 2002 / Accepted: 11 September 2002 / Published online: 11 October 2002
© SBIC 2002

Abstract Copper-64 ($T_{1/2}=12.7$ h; β^+ : 0.653 MeV, 17.4%; β^- : 0.578 MeV, 39%) is produced in a biomedical cyclotron and has applications in both imaging and therapy. Macrocyclic chelators are widely used as bifunctional chelators to bind copper radionuclides to antibodies and peptides owing to their relatively high kinetic stability. In this paper, we evaluated three tetraaza macrocyclic ligands with two, three, and four pendant methanephosphonate functional groups. DO2P [1,4,7,10-tetraazacyclododecane-1,7-di(methanephosphonic acid)], DO3P [1,4,7,10-tetraazacyclododecane-1,4,7-tri(methanephosphonic acid)], and DOTP [1,4,7,10-tetraazacyclododecane-1,4,7,10-tetra(methanephosphonic acid)] were all radiolabeled with ^{64}Cu in high radiochemical yields. Copper-64-labeled DO2P and DOTP were highly stable in rat serum out to 24 h, while ^{64}Cu -DO3P remained 73% intact, with the remainder possibly forming a $^{64}\text{Cu}_2\text{DO3P}$ dimer by 24 h. The biodistribution experiments were performed in normal Sprague-Dawley rats. Of the three complexes, ^{64}Cu -DO2P demonstrated the most optimal clearance

through the blood and liver. Copper-64-DO3P and ^{64}Cu -DOTP exhibited higher liver uptake and longer retention of liver activity, possibly because of the large negative charge of the complexes under physiological conditions. All three ^{64}Cu -labeled complexes showed high accumulation in bone, likely due to the binding of the methanephosphonate groups to hydroxyapatite. These results suggest that this series of methanephosphonate macrocyclic ligands may be useful as potential bone-imaging agents. The thermodynamic stability constants of the Cu(II) complexes with these three ligands were determined, and were found to be significantly higher than those of their acetate analogues. The Cu(II)-DO2P complex exhibited the highest stability constant among divalent transition metal ion DO2P complexes. Metabolism studies of ^{64}Cu -DO2P in rat liver suggest that the DO2P ligand may be used as a bifunctional chelator for copper radionuclides in radiodiagnostic or radiotherapeutic studies.

Electronic supplementary material is available if you access this article at <http://dx.doi.org/10.1007/s00775-002-0408-5>. On that page (frame on the left side), a link takes you directly to the supplementary material.

Electronic supplementary material is available if you access this article at <http://dx.doi.org/10.1007/s00775-002-0408-5>. On that page (frame on the left side), a link takes you directly to the supplementary material.

X. Sun · M. Wuest · Z. Wang · M. J. Welch · C. J. Anderson (✉)
The Edward Mallinckrodt Institute of Radiology,
Washington University School of Medicine,
St. Louis, MO 63110, USA
E-mail: andersoncj@mir.wustl.edu
Tel.: +1-314-3628427
Fax: +1-314-3629940

Z. Kovacs · A. D. Sherry
Department of Chemistry, University of Texas at Dallas,
Richardson, TX 75080, USA

R. Motekaitis · A. E. Martell
Department of Chemistry, Texas A&M University,
College Station, TX 77843, USA

Present address: Z. Kovacs
Macrocyclics, Inc., 17815 Davenport Rd.,
Suite 120, Dallas, TX 75252, USA

Keywords Copper-64 · Biodistribution ·
Macrocyclic · Metabolism · Thermodynamic stability

Introduction

Copper-64 ($T_{1/2}=12.7$ h; β^+ : 0.653 MeV, 17.4%; β^- : 0.578 MeV, 39%) has proven to be a versatile radionuclide with respect to its applications in both imaging [1, 2] and therapy [3, 4] and the ability to produce it on a biomedical cyclotron [5]. Macrocyclic chelators are widely used as bifunctional chelators (BFCs) to bind copper radionuclides to antibodies and peptides owing to their relatively high stability under biological conditions [6, 7, 8]. We previously demonstrated that the charge of the Cu-BFC complex attached to both monoclonal antibodies (mAbs) and peptides had a significant

effect on the clearance properties of the ^{64}Cu -BFC biomolecules [9, 10]. Additionally, it was determined that ^{64}Cu -labeled macrocyclic complexes with different formal charges showed dramatically different behavior in normal rats [11, 12]. The positively charged complexes exhibited high accumulation in the kidneys and liver out to 24 h post-injection, while neutral and negatively charged complexes similarly showed lower liver uptake and rapid clearance through kidneys [12]. In order to further evaluate Cu(II) macrocyclic complexes with differing negative charges, we determined the biological stability and in vivo behavior of ^{64}Cu complexes of DO2P [1,4,7,10-tetraazacyclododecane-1,7-di(methanephosphonic acid)], DO3P [1,4,7,10-tetraazacyclododecane-1,4,7-tri(methanephosphonic acid)], and DOTP [1,4,7,10-tetraazacyclododecane-1,4,7,10-tetra(methanephosphonic acid)] (Fig. 1). These three ligands are all 12-membered rings differing only in the number of pendant methanephosphonate functional groups.

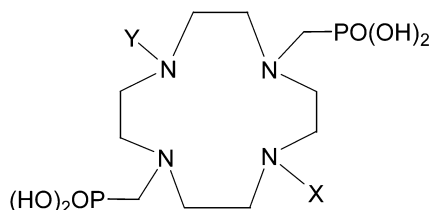
The first synthesis of DOTP was reported in 1984 [13, 14]. Since then, DOTP and its monoesters have been extensively studied for their potential applications as MRI contrast agents and clinical uses as NMR shift and relaxation reagents [1, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25] because of the structural similarity to their DOTA [1,4,7,10-tetraazacyclododecane-1,4,7,10-tetra(acetic acid)] analogues. Sherry and co-workers [26, 27] recently reported that the highly negatively charged Tm-DOTP $^{5-}$ has applications as a combined cation shift reagent and chemical marker of tissue extracellular space. Furthermore, Tm-DOTP $^{5-}$ has more rapid blood clearance than the typically less negatively charged Gd-based contrast agents and is slowly released from bone [26, 27]. In 1997, Hassfjell et al. [28] reported an α -particle emitting bone-seeking agent, ^{212}Bi -DOTP, for potential targeted radiotherapy of bone metastases; their further evaluation using ^{205}Bi -DOTP (a γ -emitter) revealed that ^{205}Bi -DOTP was deposited heterogeneously in bone with the highest concentration in the bone matrix and was cleared in the urine as an intact complex

[29]. Recently, Kothari et al. [30] reported the evaluation of ^{186}Re -labeled 1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetra(methanephosphonic acid) for possible use in metastatic bone-pain palliation.

A number of derivatives of tetraazacyclododecanes with pendant methanephosphonate functional groups have been synthesized and their thermodynamic stability, dissociation kinetics, metal selectivity, and coordination behavior with divalent and trivalent transition metal ions and lanthanide ions have been examined [31, 32, 33]. Among the divalent metal ions studied (Mg^{2+} , Ca^{2+} , Sr^{2+} , Mn^{2+} , and Zn^{2+}), Zn(II) formed the most stable complex with DO2P [$\log K_{\text{Zn(II)L}} = 21.2$ (25 °C, 0.1 M KCl)].

There are few reports on the Cu(II) complexes of these methanephosphonate macrocyclic ligands, although the stability constant of Cu(II)-DOTP was reported [$\log K_{\text{Cu(II)DOTP}} = 25.4$ (1 M KNO_3); $\log K_{\text{Cu(II)DOTA}} = 22.3$ for comparison] [13, 14]. By electronic absorption spectrophotometry and electron paramagnetic resonance (EPR) spectroscopy, the solution structure of the Cu(II)-DOTP complex was recently determined to be a square-pyramidal geometry with four ring-nitrogen atoms defining the equatorial plane, while the axial position is probably occupied by an oxygen from one methanephosphonate side-arm [34]. This structure is similar to the solid state structure of the Cu(II) complex with a similar ligand, *N,N',N'',N'''*-tetrakis[2-(diphenylphosphoryl)ethyl]-1,4,7,10-tetraazacyclododecane [35]. A derivative of cyclen with three methylene(phenyl)phosphonic acid pendant side-arms [1,4,7,10-tetraazacyclododecane-1,4,7,10-tetramethyltrimethylenetris(phenylphosphonic acid)], similar to DO3P, was reported by Lukes et al. [32] and Rohovec et al. [36, 37]. In the solid state, this ligand forms a dimer complex ($[\text{LnL}]_2$) with seven different lanthanide(III) ions (Ln = La, Ce, Nd, Eu, Tb, Er, Yb) with phosphonic acid as a bridging functional group. DO3P has not previously been reported as a ligand for either transition metal ions or lanthanide(III) ions. To the best of our knowledge, the biological behavior of DO2P and DO3P metal ion complexes have not yet been evaluated.

In this paper, the radiochemistry, in vitro and in vivo stability, and biodistribution data of ^{64}Cu -labeled DO2P, DO3P, and DOTP complexes are presented.



DO2P: X, Y = H;
DO3P: X = H, Y = $\text{CH}_2\text{PO}(\text{OH})_2$;
DOTP: X, Y = $\text{CH}_2\text{PO}(\text{OH})_2$

Fig. 1 Structures of three tetraazacyclododecane ligands with methanephosphonate functional groups: DO2P [1,4,7,10-tetraazacyclododecane-1,7-di(methanephosphonic acid)], DO3P [1,4,7,10-tetraazacyclododecane-1,4,7-tri(methanephosphonic acid)], and DOTP [1,4,7,10-tetraazacyclododecane-1,4,7,10-tetra(methanephosphonic acid)]

Materials and methods

Reagents and instrumentation

Copper-64 was prepared on the Washington University Medical School Cyclotron CS-15 by the $^{64}\text{Ni}(p,n)^{64}\text{Cu}$ nuclear reaction at a specific activity of 50–300 mCi/ μg as previously described [5]. Water was distilled and then deionized (18 M Ω /cm 2) by passing through a Milli-Q water filtration system (Millipore, Bedford, Mass., USA). Ammonium acetate was purchased from Fluka (Buchs, Switzerland). DOTA was purchased from Strem (Newburyport, Mass., USA), and DO2A [1,4,7,10-tetraazacyclododecane-1,7-di(acetic acid)] was prepared as previously described [38]. Rat serum was purchased from Sigma (St. Louis, Mo., USA).

Waters C18 silica gel thin layer chromatography (TLC) plates (KC18F, 60 Å, 200 µm) were purchased from Fisher (Pittsburgh, Pa., USA). Radio-TLC was performed using a Bioscan 200 imaging scanner (Bioscan, Washington, DC, USA). Radioactivity was counted with a Beckman Gamma 8000 counter containing a NaI crystal (Beckman, Irvine, Calif., USA).

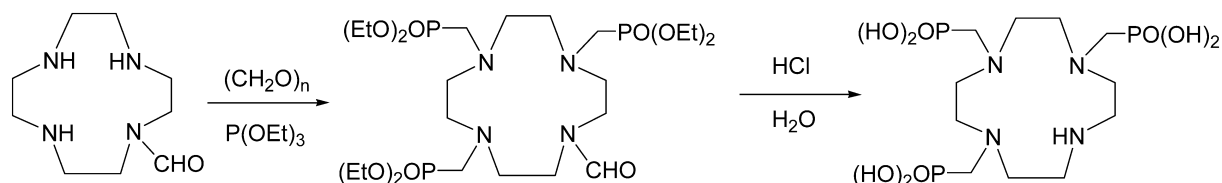
The ^1H and ^{13}C NMR spectra were recorded on a 500 MHz Varian Unity INOVA spectrometer using an external reference. Chemical shifts were relative to TMS. FT-IR spectra were recorded on a Nicolet Avatar 360 FT-IR spectrometer. Elemental microanalysis was performed by Oneida Research Services (Whitesboro, NY, USA). The mass spectra were recorded on a Waters ZQ mass spectrometer equipped with an ESI probe operated under a positive polarity.

Complex charge was determined by electrophoresis using a Helena Laboratories electrophoresis chamber (Beaumont, Tex., USA) with Sephaphore III cellulose polyacetate strips (Gelman, Ann Arbor, Mich., USA) presoaked in 0.4 M ammonium acetate buffer at either pH 5.5 or 7.4. The strips were developed using a Bio-Rad model 1000/500 power supply (Richmond, Calif., USA) at a constant current of 50 mA and a power of 10 W for 120 min. The ^{64}Cu -DOTA and ^{64}Cu -DO2A complexes, known to have overall charges of 2- and neutral, respectively, were used as standards [12]. The strips were analyzed using the Bioscan 200 imaging scanner to determine the migration of radioactivity and overall charge of the complexes.

Ligand synthesis

DO2P and DOTP were prepared by literature methods [39, 40]. DO3P was synthesized as follows (Scheme 1). A 500-mL round-bottom flask was loaded with *N*-formylcyclen (1-formyl-1,4,7,10-tetraazacyclododecane) (5.00 g) and triethyl phosphite (15.00 g, 20% excess) and the flask was immersed in an ice bath [41]. Paraformaldehyde (2.47 g, 10% excess) was added in small portions over a period of 30 min. The mixture was then allowed to warm up to room temperature and stirring was continued for 2 days at room temperature and 1 day at 40 °C. The clear mixture was kept under high vacuum at 40–50 °C for several hours to remove volatile impurities. The residual crude phosphonate ester was hydrolyzed without further purification. It was dissolved in hydrochloric acid (20%, 200 mL) and the mixture was refluxed for 2 days. The hydrochloric acid was removed by rotary evaporation to give a clear oil. It was dissolved in water (100 mL) and the solvent was removed by rotary evaporation. This procedure was repeated two more times, when the product separated as fine white crystals. It was filtered off, washed with water (3×50 mL), and air-dried. The crude product (12.26 g) was recrystallized from hot water to give a white crystalline solid which was dried in vacuum to constant mass. Yield: 9.26 g (80.0%). ^1H NMR [500 MHz, D_2O , NH_3 , pH 10, δ (ppm)]: 2.67 (d, $J_{\text{PH}} = 11$ Hz, 4H), 3.06 (br s, 12H), 3.14 (d, $J_{\text{PH}} = 11$ Hz, 2H), 3.35 (br s, 4H); ^{13}C NMR [125 MHz, D_2O , NH_3 , pH 10, δ (ppm)]: 44.65, 51.36 (d, $J_{\text{PC}} = 5.5$ Hz), 51.53 (d, $J_{\text{PC}} = 4.7$ Hz), 52.51, 52.54 (d, $J_{\text{PC}} = 128.6$ Hz), 54.05 (d, $J_{\text{PC}} = 136.5$ Hz); FT-IR [KBr, λ (cm^{-1})]: 3543, 3093, 2960, 2930, 2869, 1651, 1511, 1462, 1412, 1215, 1170, 1029, 962, 883, 825, 734, 553, 530, 423; MS [(ESI) m/z]: calcd for DO3P: 454; found: 455.10 ($M + \text{H}^+$, 100%); anal. calcd for $\text{C}_{11}\text{H}_{29}\text{N}_4\text{O}_9\text{P}_3 \cdot 0.50\text{H}_2\text{O}$: C, 28.52; H, 6.36; N, 12.04; found: C, 28.52; H, 6.42; N, 12.09.

Scheme 1



Stability measurements

The determination of the stability constants of Cu(II)-DO2P and Cu(II)-DO3P is presented here. Although the stability constant of Cu(II)-DOTP has been reported elsewhere [13, 18], it was also measured in this study for comparison.

The protonation constants were determined by ordinary potentiometric titration as described [42]. Computations of protonation constants of these three ligands were done using program BEST [42]. The stability constants of their Cu(II) complexes were determined by potentiometric titration. The pH measurements were performed with a Fisher Accumet 925 pH meter, an Orion 8103 Ross Combination electrode, and a Metrohm automatic burette in a jacketed vessel at 25.0 ± 0.1 °C under a nitrogen atmosphere. The ionic strength was kept constant with 0.1 M KCl. Hydrogen ion concentrations were calculated from the measured pH values using $\text{p}K_w = 13.806$ and a H^+ activity coefficient of 0.82, determined in separate titrations. The stability constants were obtained from the potentiometric data by using a Simplex nonlinear regression algorithm run on a PC [43]. All measurements were in triplicate.

Preparation of ^{64}Cu -labeled DOTA and DO2A complexes

Ligand solutions (5 mM in 0.4 M ammonium acetate, pH 5.5) were prepared by dissolving the ligand solids in 0.4 M ammonium acetate buffer. To 100 µL of a ligand solution, ^{64}Cu chloride (0.5 µL in 0.1 M HCl, ca. 1.2 mCi) was added. The formation of both complexes was complete within 2 h at RT as determined by radio-TLC on silica, eluting with 1:1 methanol/10% ammonium acetate. Freshly prepared ^{64}Cu -labeled DOTA and DO2A complexes were employed as electrophoresis standards [12].

Preparation of the ^{64}Cu -ligand complexes

Ligand solutions (2.0–5.0 mM) were prepared by dissolving DO2P, DO3P, or DOTP in either 0.4 M or 0.1 M ammonium acetate, pH 6.5. Copper-64 chloride (1–2 µL in 0.1 M HCl, 3–6 mCi) was converted to ^{64}Cu acetate by stirring with 0.4 M ammonium acetate (50 µL), pH 6.5. Then ^{64}Cu acetate (< 5 µL, 0.1–0.5 mCi) was added to the ligand solutions (100 µL, 2.0–5.0 mM). Reaction times ranged from 1 to 24 h and temperatures ranged from room temperature to 90 °C (Table 1). A microwave cavity with a power of ca. 180 W was employed to speed up the radiolabeling of DO2P. The radiochemical purity was determined by reversed-phase TLC. The eluant composition for developing ^{64}Cu -labeled DO2P and DO3P complexes was MeOH/10% ammonium acetate (1:4), and for ^{64}Cu -DOTP complex the eluant composition was MeOH/10% ammonium acetate (1:9). Under both conditions, ^{64}Cu acetate remained at the origin.

Determination of partition coefficients

The partition coefficients ($\log P$) of the ^{64}Cu (II) complexes were determined by adding 4–8 µL of the labeled complex (5.0 mM) to a solution containing 500 µL of octanol and 500 µL of Milli-Q water (obtained from saturated octanol/water solutions) ($n = 5$). The resulting solutions were then shaken for 2 h at RT. From each of the five samples, aliquots of 400 µL and 50 µL were removed from the octanol phase and the water phase, respectively, and counted separately. The partition coefficient was calculated as the ratio of

Table 1 Labeling conditions for $^{64}\text{Cu}(\text{II})$ -labeled DO2P, DO3P, and DOTP under no-carrier-added (NCA) or carrier-added (CA) levels, partition coefficients, and their electrophoresis behavior. In all reactions, $<5\ \mu\text{L}$ ^{64}Cu -acetate was added to $100\ \mu\text{L}$ of the ligand solution in $0.4\ \text{M}$ or $0.1\ \text{M}$ ammonium acetate (2.0 – $5.0\ \text{mM}$,

pH 6.5). The TLC conditions were C18 plates as the stationary phase and $1:4\ \text{MeOH}/10\%$ ammonium acetate as the eluant, except for ligand DOTP, where the eluant was $1:9\ \text{MeOH}/10\%$ ammonium acetate

Ligand	Reaction conditions	Radiochemical purity (%)	R_f	$\log P$	Electrophoresis d (mm) ^a
DO2P	NCA: 16 h at 70 °C or 4 h at 90 °C or 90 s at ca. 180 W ^b CA: 2 h at RT	97.8 99.7 ~100	0.42	-2.67 ± 0.12	8.4
DO3P	NCA: 2 h at RT	96.6	0.31	-3.02 ± 0.21	11.6
DOTP	NCA: 2 h at RT	96.9	0.57	-2.89 ± 0.22	13.8
DO2A	NCA: 2 h at RT	100	0.15 ^c	n.d. ^d	3.4
DOTA	NCA: 2 h at RT	99.9	0.40 ^c	n.d.	13.5

^aThe migration distance in the direction of the anode (+)

^bIn a microwave cavity

^cSilica; 1:1 methanol/10% ammonium acetate

^dn.d.: not determined

counts in the octanol fraction to the counts in the water fraction (multiplied by 8). An average $\log P$ value was obtained from the five samples.

Serum stability

In vitro serum stability experiments were conducted by adding $50\ \mu\text{L}$ of ^{64}Cu -labeled DO2P, DO3P, and DOTP complexes to $500\ \mu\text{L}$ of rat serum. The solutions were incubated at $37\ ^\circ\text{C}$, and samples were analyzed by radio-TLC at 10, 30, and 60 min, and 2, 4, and 24 h post-administration to rat serum.

Biodistribution studies

All animal studies were performed in compliance with guidelines set by the Washington University Animal Studies Committee. Copper-64-labeled DO2P, DO3P, and DOTP solutions were diluted with saline. Mature female Sprague-Dawley rats ($n=4$ per time point) weighing 180 – $200\ \text{g}$ were anesthetized with isoflurane and injected with ca. $22\ \mu\text{Ci}$ of activity via the tail vein. The injected volume of activity per rat did not exceed $0.2\ \text{mL}$. The rats were anesthetized prior to sacrifice (by decapitation) at each time point. Organs of interest were removed, weighed, and counted. 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).

Metabolism studies

The metabolism of ^{64}Cu -DO2P in rat liver in vivo was performed in mature female Sprague-Dawley rats using previously reported methods [44]. Briefly, ^{64}Cu -DO2P was injected into rats via the tail vein. The rats were sacrificed at 1 h, 4 h, and 24 h post-injection, and the livers were immediately excised and placed on ice. Tissue samples were homogenized in $65:35$ ethanol/ammonium acetate buffer ($0.1\ \text{M}$, pH 5.5) followed by a 1-min sonication. The precipitated protein was separated by centrifugation at $23,500g$ for 30 min at $4\ ^\circ\text{C}$. Liver controls were performed where the ^{64}Cu -DO2P injectate was added directly to liver tissue excised from rats prior to homogenization and centrifugation. These samples were worked up in the same manner as the experimental rats as described above. All liver supernatants were analyzed by fast protein liquid chromatography (FPLC). Briefly, a $100\text{-}\mu\text{L}$ aliquot of the supernatant was injected on a Superose-12 gel filtration column (calibrated using molecular weight standards), which was then eluted with $20\ \text{mM}$ Hepes and $300\ \text{mM}$ NaCl (pH 7.3) buffer at a flow rate of $0.5\ \text{mL}/\text{min}$. The fractions were counted on a γ

counter. To ensure that no activity was neglected, the activity of fractions collected at all time points was compared to the amount injected. Greater than 95% of the radioactivity was recovered from the column. The percent authentic intact (%AI) was calculated as previously described [45]. An unpaired t -test on the metabolism data was performed using Prism, v. 3.00 (Graphpad, San Diego, Calif., USA).

Results

Synthesis

DO3P was synthesized by reacting the monoprotected cyclen derivative 1-formylcyclen [41] with 3 equiv of paraformaldehyde and triethyl phosphite as described for DO2P [31]. Acid hydrolysis of the phosphonate bis(ethyl ester) in hydrochloric acid cleaved both the phosphonate ester groups and the N -formyl protection. The product crystallized out of slightly acid solution and recrystallization from water resulted in a pure product. No impurities were detected by high-resolution ^1H or ^{13}C NMR. The structure was also confirmed by IR and MS data. Elemental microanalysis showed that the compound crystallized with $0.5\ \text{mol}$ of water of crystallization and did not contain hydrochloric acid.

Radiochemistry

The reaction conditions, TLC conditions, and ^{64}Cu -labeling yields are presented in Table 1. At the no-carrier-added (NCA) level (the ratio of $^{64}\text{Cu}/\text{L}$ is about $10^{-5}:1$), DO3P and DOTP ($2.0\ \text{mM}$ – $5.0\ \text{mM}$ each) were successfully labeled with ^{64}Cu after 2 h at RT in $0.4\ \text{M}/0.1\ \text{M}$ ammonium acetate (pH range from 5.5 to 8.5) in high radiochemical purity ($>96\%$). Copper-64-DO2P was prepared after 16 h at $70\ ^\circ\text{C}$ or 4 h at $90\ ^\circ\text{C}$ under the same conditions in yields of $>97\%$. DO2P was also radiolabeled with Cu-64 (NCA) in a microwave cavity (ca. $180\ \text{W}$) for 90 s in 99.7% yield. At the carrier-added

(CA) level [the ratio of Cu(⁶⁴Cu)/L is from 10⁻¹:1 to 1:1], DO2P was labeled in quantitative yield after 2 h at RT.

Copper-64-labeled complexes were analyzed by electrophoresis to determine the charge of the complexes (Table 1). The migration distances of the ⁶⁴Cu complexes were compared with the migration distances of ⁶⁴Cu-DOTA (2- charge) and ⁶⁴Cu-DO2A (neutral) standards [12]. At pH 5.5 (0.4 M ammonium acetate), the ⁶⁴Cu-DOTA and ⁶⁴Cu-DO2A standards migrated with distances of 13.5 mm and 3.35 mm, respectively, in the direction of the anode. The slight migration of the ⁶⁴Cu-DO2A standard toward the anode was due to electroosmotic transport [46], and is consistent with its neutral charge. Under the same experimental conditions, the ⁶⁴Cu-DO2P, -DO3P, and -DOTP complexes migrated with distances of 8.4, 11.6, and 13.8 mm, respectively, in the direction of the anode, indicating a negative charge for all three complexes in the ammonium acetate buffer, pH 5.5. The same results were obtained at physiological pH values (0.4 M ammonium acetate, pH 7.4).

The octanol-water partition coefficients or log *P* values of the ⁶⁴Cu-DO2P, -DO3P, and -DOTP complexes were determined to be -2.67 ± 0.12, -3.02 ± 0.21, and -2.89 ± 0.22, respectively, indicating that they are all hydrophilic complexes (Table 1).

In the in vitro serum stability experiments, the concentration of ligand was in the range 0.33–0.83 mM, while the protein concentration in rat serum for copper was in large excess. These experiments showed by radio-TLC that ⁶⁴Cu-labeled DO2P and DOTP remained nearly 100% intact, while the ⁶⁴Cu-DO3P complex was approximately 73% intact in rat serum at 24 h post-administration. These data demonstrated high in vitro stability of the ⁶⁴Cu complexes and suggested that they were worthy of in vivo investigation.

Protonation and stability constants

The log protonation constants of DO2P measured in this work are identical to those previously reported [31], and the log protonation constants of DOTP determined here are very similar to those previously reported [18]. The stepwise log protonation constants of DO3P are 12.9, 11.4, 8.69, 7.09, 5.53, and 1.42.

Thermodynamic stability constants for the Cu(II) complexes of these three ligands and their acetate

analogues are presented in Table 2. The stability constant of Cu(II)-DOTP measured here (26.2 ± 0.2) is about the same as the value previously reported (25.4) [13, 34]. The stability constants of Cu(II)-DO2P and Cu(II)-DO3P are 28.7 ± 0.3 and 26.9 ± 0.2, respectively. Compared to their acetate analogues, the methanephosphonate cyclen ligands have significantly higher stability constants when complexed with Cu(II). The Cu(II)-DO2P complex exhibited the highest stability constant among the DO2P complexes with the divalent metal ions [31, 32].

Biodistribution experiments

The biodistribution results of the three ⁶⁴Cu-labeled complexes in major organs of interest are presented in Fig. 2. Copper-64-DO2P showed a high bone uptake within 30 min post-injection [1.21 ± 0.16%ID/g (22.50 ± 2.16%ID/bone) at 5 min and 1.19 ± 0.12%ID/g (23.12 ± 2.67%ID/bone) at 30 min], which decreased over time to 0.46 ± 0.08%ID/g (8.88 ± 1.82%ID/bone) at 2 h and 0.17 ± 0.01%ID/g (3.43 ± 0.29%ID/bone) at 24 h. A rapid blood clearance was observed from 5 min (1.73 ± 0.21%ID/g) out to 2 h (0.04 ± 0.01%ID/g), with further clearance out to 24 h post-injection (0.01 ± 0.002%ID/g). The rapid blood clearance of ⁶⁴Cu-DO2P in vivo suggests that the complex did not appreciably dissociate in the blood. Significant uptake was observed initially for liver and kidney (0.30 ± 0.06%ID/g and 4.22 ± 1.01%ID/g at 5 min, respectively) that decreased over time (0.07 ± 0.01%ID/g and 0.28 ± 0.07%ID/g at 24 h, respectively). No significant lung, spleen, heart, or brain uptake was observed. The uptake in the clearance organs showed that ⁶⁴Cu-DO2P cleared rapidly through the kidneys, while a relatively small amount cleared via the liver into the intestines.

The ⁶⁴Cu-DO3P complex exhibited very high bone uptake within 30 min post-injection [2.05 ± 0.27%ID/g (42.34 ± 7.03%ID/bone) at 5 min and 2.73 ± 0.09%ID/g (55.87 ± 1.54%ID/bone) at 30 min], and slow clearance [1.74 ± 0.15%ID/g (35.76 ± 4.17%ID/bone) at 24 h]. The blood, liver, and kidney accumulation decreased over time (0.85 ± 0.12%ID/g, 0.90 ± 0.10%ID/g, and 3.78 ± 0.82%ID/g at 5 min, respectively; 0.10 ± 0.01%ID/g, 0.59 ± 0.09%ID/g, and 1.22 ± 0.32%ID/g at 24 h, respectively). No significant lung, spleen, heart, or brain uptake was observed.

Table 2 Stability constants for Cu(II) complexes of DO2P, DO3P, DOTP, and their corresponding acetate analogues. Stability constants are defined as $K_{M(II)L} = [M(II)L]/[M(II)][L]$ (in units of M⁻¹)

	DO2P	DO3P	DOTP	DO2A	DO3A	DOTA
log $K_{Cu(II)L}$	28.7 ± 0.3 ^a	26.9 ± 0.2 ^a	25.4 ^b 26.2 ± 0.2 ^a	18.9 ^c	22.87 ^d	22.3 ^e

^aThis work (25 °C, 0.1 M KCl)

^bFrom refs. [14, 34] (25 °C, 1.0 M KNO₃)

^cFrom ref. [12] (25 °C, 0.1 M KCl)

^dFrom ref. [50] (25 °C, 0.1 M Me₄NNO₃)

^eFrom refs. [31, 51, 52] (25 °C, 0.1 M Me₄NNO₃)

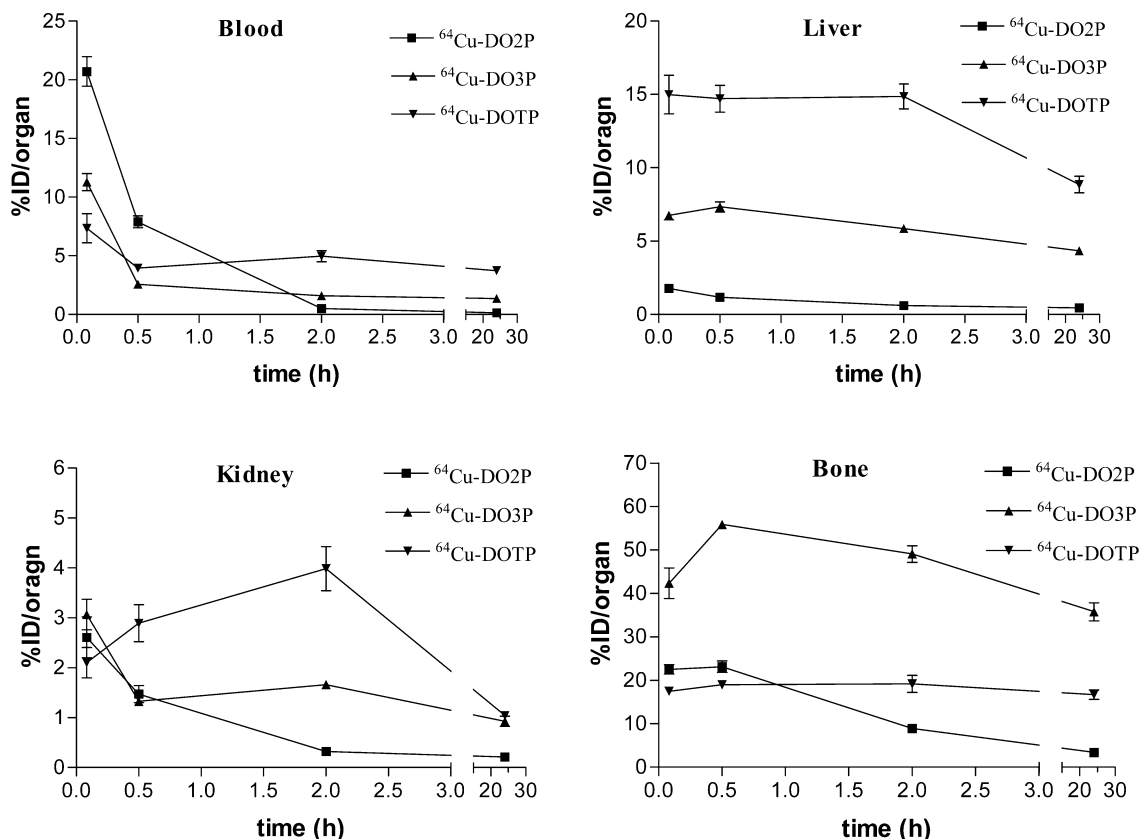


Fig. 2 Rat biodistribution data for three ^{64}Cu -labeled complexes in organs of interest (blood, liver, kidney, and bone). Data are presented as %ID/organ versus time ($n=4$ for each time point)

The biodistribution results of ^{64}Cu -labeled DOTP demonstrated relatively high uptake and minimal clearance for bone and liver [$0.93 \pm 0.12\% \text{ID/g}$ ($17.45 \pm 2.00\% \text{ID/bone}$) and $2.25 \pm 0.49\% \text{ID/g}$ ($16.69 \pm 2.11\% \text{ID/bone}$) and $1.14 \pm 0.08\% \text{ID/g}$ at 24 h, respectively]. The ^{64}Cu -DOTP uptake in blood and kidney also cleared slowly ($0.61 \pm 0.22\% \text{ID/g}$ and $2.94 \pm 0.86\% \text{ID/g}$ at 5 min, respectively; $0.31 \pm 0.03\% \text{ID/g}$ and $1.35 \pm 0.23\% \text{ID/g}$ at 24 h, respectively). No significant lung, spleen, heart, or brain uptake was observed.

Metabolism

To further verify the potential of DO2P as a BFC for labeling copper radionuclides to biological molecules, metabolism studies were carried out in normal rats using reported methods [44] to determine the extent of ^{64}Cu transchelation to proteins observed after injection of ^{64}Cu -DO2P. The percent authentic intact (%AI) of ^{64}Cu -DO2P at 1 h, 4 h, and 24 h post-injection was calculated as previously described [44, 45]. The major ^{64}Cu -labeled protein present had a MW of 32 kDa (^{64}Cu -SOD), consistent with that previously reported [44]. Copper-64-DO2P remained $37.24 \pm 1.46\%$, $30.96 \pm 1.59\%$, and $21.06 \pm 6.75\%$ intact in rat liver at

1 h, 4 h, and 24 h post-injection, respectively ($n=3$). At 24 h post-injection, it was significantly more stable than ^{64}Cu -TETA ($7.78 \pm 5.41\%$ intact at 20 h post-injection; P value of the unpaired t -test: 0.0215) [47].

Discussion

In this study we evaluated a series of tetraaza macrocyclic ligands that differed in the number of methanephosphonate functional groups. The purpose of this work was to determine the effects of the number of methanephosphonate groups of ^{64}Cu -labeled complexes on the in vivo and in vitro stability and biodistribution of the ^{64}Cu complexes. One of the goals was to determine if the three ligands might be potential candidates for BFCs for complexing copper radionuclides to biological molecules, while another goal was to evaluate the complexes as potential bone-imaging agents.

The three methanephosphonate macrocyclic ligands were labeled with ^{64}Cu under conditions shown in Table 1. Copper-64-DO2P, which had the highest thermodynamic stability constant, required a longer labeling time and higher temperature at the NCA level. This likely reflects the experimental conditions for this experiment. At very low concentrations of ^{64}Cu (the ratio of $^{64}\text{Cu}/\text{L}$ was about $10^{-5}:1$) used in the radiolabeling experiment and for a bimolecular reaction, one would expect a slow complex formation for the ligand with the least negative charge, which was observed for

^{64}Cu -DO2P. However, at the CA level (also at the higher concentrations used in the potentiometric study), such slow reaction kinetics were not observed (Table 1). A microwave cavity was employed to overcome the slow kinetics of radiolabeling DO2P with ^{64}Cu , with positive results (Table 1). The rapid quantitative reaction under the assistance of the microwave may provide a fast, feasible method for labeling DO2P and its BFC-peptide conjugates.

The hydrophilic nature of the complexes was reflected in the negative $\log P$ values (Table 1). While the hydrophilicity of ^{64}Cu -DO3P and ^{64}Cu -DOTP did not differ significantly, both are somewhat more hydrophilic than ^{64}Cu -DO2P. This likely reflects the additional uncoordinated methanephosphonate groups in the DO3P and DOTP complexes.

The electrophoresis results for the ^{64}Cu -labeled complexes of DO2P, DO3P, and DOTP (Table 1) indicated that they were all negatively charged in ammonium acetate buffers of pH 5.5 and physiological pH values (pH 7.4). Compared to the migration distances of ^{64}Cu -DOTA (13.0 mm, 2- charge) and ^{64}Cu -DO2A (3.4 mm, neutral) in the direction of anode, the migration distances of ^{64}Cu -labeled DO2P (8.4 mm), DO3P (12.6 mm), and DOTP (13.8 mm) complexes suggest that they became more negatively charged as the number of methanephosphonate functional groups increased. However, the net charge on a complex may not necessarily be quantified by its migration distance in the electrophoresis experiments, because different complex ions may have different ionic mobility that is related to their individual radius, ionic atmosphere, and charge [48].

The stability constants of their Cu(II) complexes are reported in Table 2. Of the three Cu(II) complexes, Cu-DO2P had the highest stability constant ($\log K_{\text{ML}} = 28.7$) by nearly two orders of magnitude over Cu-DO3P ($\log K_{\text{ML}} = 26.9$). The stability constants of the Cu(II) complexes of all these methanephosphonate macrocyclic ligands were several orders of magnitude higher in thermodynamic stability compared to their acetate analogues, with the greatest difference being between Cu-DO2P ($\log K_{\text{ML}} = 28.7$) and Cu-DO2A ($\log K_{\text{ML}} = 18.9$). This observation is consistent with what Lukes et al. [32] reported on the order of thermodynamic stability of Cu(II) complexes formed with cyclam and cyclen derivatives containing methanephosphonic, methanephosphinic, and acetic acid arms. Of the stability constants of the Cu(II) macrocyclic ligands reported in the literature [49], Cu-DO2P is one of the highest. Nevertheless, the value found was somewhat unexpected because Cu-DO2P is more stable than either Cu-DO3P or Cu-DOTP. This same trend was not observed for DO2A, DO3A, and DOTA. It has been reported that the Cu^{2+} in Cu-DOTA is hexacoordinate (two coordinated and two uncoordinated carboxylates) while the Cu^{2+} in Cu-DOTP is pentacoordinate (one coordinated phosphonate) [31, 34]. This difference likely reflects the fact that it is more difficult to get two more

highly charged methanephosphonates bound to a Cu^{2+} in *cis* positions (as required here) than two carboxylates, even though methanephosphonate oxygens are more basic than carboxylate oxygens. Taken together, these data suggest that Cu-DO2P has a different structure in solution than either Cu-DO3P or Cu-DOTP. While Cu-DOTP has been shown to be pentacoordinate with one coordinated phosphonate oxygen [34], our stability data suggests that Cu-DO2P is most likely hexacoordinate. Proof of this will require further optical studies or a crystal structure of the complex.

Because of the lability of Cu(II) to ligand exchange, thermodynamic stability is often not an accurate indicator of *in vivo* stability. The latter is more determined by the kinetic stability of the complexes [8]. For this reason we compared the thermodynamic stability constants with biological stability of the ^{64}Cu -labeled complexes in serum and *in vivo*.

The *in vitro* serum stability experiments showed both ^{64}Cu -DO2P and ^{64}Cu -DOTP remained 100% intact out to 24 h post-administration. The result for ^{64}Cu -DOTP is consistent with previously reported studies on DOTP metal ion complexes [26, 27, 29]. The high *in vitro* stability of ^{64}Cu -DO2P is possibly because it may have a hexacoordinate structure as described above. Although ^{64}Cu -DO3P was approximately 73% intact in rat serum at 24 h post-administration, it still exhibited comparable *in vitro* stability as compared to most of the tetraaza macrocyclic ligands evaluated [11, 12]. Furthermore, the additional species that appeared on the radio-TLC was not free ^{64}Cu or ^{64}Cu -associated proteins, as indicated by its R_f value (ca. 0.7). This implies that ^{64}Cu was not transchelated or decomposed from ^{64}Cu -DO3P. Our assumption is that a ^{64}Cu -2-DO3P dimer is formed, since DO3P has an additional methanephosphonate side-arm and the ligand was in large excess. A similar phenomenon was observed in a recently published study by our group [47]. Thus, it is reasonable to assume that ^{64}Cu -DO3P was stable to ^{64}Cu dissociation in rat serum out to 24 h post-administration.

The *in vivo* behavior of ^{64}Cu -labeled complexes of DO2P, DO3P, and DOTP is shown in Fig. 2. An increase in the number of side-chain methanephosphonate groups dramatically altered the biodistribution of the ^{64}Cu complexes. Of the three complexes, ^{64}Cu -DO2P demonstrated the most efficient clearance through blood, liver, and kidney, ^{64}Cu -DOTP exhibited the longest retention in the tissues, while the DO3P complex was intermediate in both uptake and clearance. The rat biodistribution data for ^{64}Cu -DO2P (the complex with least negative charge) are consistent with what has been previously reported for the ^{64}Cu -labeled aza macrocyclic complexes of DO2A and TETA. Jones-Wilson et al. [12] reported that positively charged ^{64}Cu -labeled cyclam and Et-cyclam had higher accumulations in the liver of normal rats, while the negatively charged and neutral ^{64}Cu -labeled tetraaza cyclic complexes cleared significantly through the liver and kidney by 24 h

Table 3 Comparison of rat biodistribution data for ^{64}Cu -DO2P to ^{64}Cu -labeled related complexes at 24 h post-injection. Data are presented as %ID/organ \pm s.d.

Ligand	Blood	Liver	Kidney
TETA ^a	0.21 \pm 0.05	0.49 \pm 0.11	0.21 \pm 0.03
Cyclen ^a	1.73 \pm 0.14	3.90 \pm 0.40	2.14 \pm 0.89
DOTA ^a	0.58 \pm 0.19	1.05 \pm 0.16	0.54 \pm 0.08
DO2A ^a	0.46 \pm 0.08	0.86 \pm 0.14	0.35 \pm 0.10
DO2P	0.13 \pm 0.02	0.45 \pm 0.06	0.21 \pm 0.06

^aFrom ref. [12]

post-injection. Compared to other ^{64}Cu -labeled cyclen derivatives, e.g. DO2A and DOTA, ^{64}Cu -DO2P exhibited more rapid clearance through blood, liver, and kidney at 24 h post-injection (Table 3). Copper- ^{64}Cu -DO2P had very similar uptake and clearance from blood, liver, and kidney as ^{64}Cu -TETA, the most widely used BFC for labeling copper radionuclides to biological molecules. This again adds support to our suggestion that Cu-DO2P, like Cu-TETA, is hexacoordinate. Rapid clearance of ^{64}Cu -DO2P from the liver was indicative of its high in vivo stability, which was further confirmed by the metabolism studies of ^{64}Cu -DO2P in rat liver. All these data strongly support that DO2P may be used as a BFC for copper radiopharmaceuticals.

The significant accumulation of the three ^{64}Cu -labeled complexes in bone (Fig. 2) is expected, based on their structural characteristics of pendant methanephosphonate functional groups that likely bind to hydroxyapatite in the bone. However, this bone activity may not be a limiting factor in the use of the methanephosphonate macrocyclic derivatives as BFCs, because once attached to an antibody they may lose their bone-seeking properties. One might take advantage of the bone-seeking properties of these low molecular weight complexes by using ^{64}Cu - and ^{61}Cu -labeled DO2P, DO3P, and DOTP as PET bone-imaging agents. Such studies are in progress.

Conclusions

The thermodynamic stability of the Cu(II) complexes of DO2P, DO3P, and DOTP and the radiochemistry and biological behavior of ^{64}Cu -DO2P, -DO3P, and -DOTP have been evaluated. The thermodynamic stability of the Cu(II) methanephosphonate ligands compared favorably to the Cu(II) complexes of their acetate analogues, with Cu(II)-DO2P having the highest stability constant ($\log K_{\text{ML}} = 28.7$). All of the ^{64}Cu complexes had considerable uptake in the bone, which makes them potential PET bone imaging agents, as well as bone palliation and therapy agents. Of the three ^{64}Cu -labeled complexes, ^{64}Cu -DO2P had the most favorable biodistribution in normal rats, with very rapid clearance through the blood, liver, and kidney; it also showed high resistance to transchelation. Taken together, the ligand DO2P has the most promise as a

BFC for labeling copper radionuclides to biological molecules.

Acknowledgements The authors would like to thank Lynne Jones and Nicole Mercer for technical assistance, Paul McQuade for the analysis of the mass spectra, and Carmen Dence for the assistance in use of the microwave. The production of ^{64}Cu at Washington University is supported by a grant from the National Cancer Institute (1-R24-CA86307; M.J.W.). The authors acknowledge funding by NIH grants (CA42925, M.J.W.; CA93375, C.J.A.), the Robert A. Welch Foundation (AT-584; A.D.S.), and the NIH (RR-02584; A.D.S.).

References

- Aime S, Ascenzi P, Comoglio E, Fasano M, Paoletti S (1995) *J Am Chem Soc* 117:9365–9366
- Philpott GW, Schwarz SW, Anderson CJ, Dehdashti F, Connett JM, Zinn KR, Meares CF, Cutler PD, Welch MJ, Siegel BA (1995) *J Nucl Med* 36:1818–1824
- Anderson CJ, Jones LA, Bass LA, Sherman ELC, McCarthy DW, Cutler PD, Lanahan MV, Cristel ME, Lewis JS, Schwarz SW (1998) *J Nucl Med* 39:1944–1951
- Connett JM, Anderson CJ, Guo LW, Schwarz SW, Zinn KR, Rogers BE, Siegel BA, Philpott GW, Welch MJ (1996) *Proc Natl Acad Sci USA* 93:6814–6818
- McCarthy DW, Shefer RE, Klinkowstein RE, Bass LA, Margenau WH, Cutler CS, Anderson CJ, Welch MJ (1997) *Nucl Med Biol* 24:35–43
- Cole WC, DeNardo SJ, Meares CF, McCall MJ, DeNardo GL, Epstein AL, O'Brien HA, Moi MK (1987) *J Nucl Med* 28:83–90
- Chaves S, Delgado R, Frausto da Silva JJR (1992) *Talanta* 39:249–254
- Moi MK, Meares CF, McCall MJ, Cole WC, DeNardo SJ (1985) *Anal Biochem* 148:249–253
- Anderson CJ, Pajean TS, Edwards WB, Sherman EL, Rogers BE, Welch MJ (1995) *J Nucl Med* 36:2315–2325
- Rogers BE, Anderson CJ, Connett JM, Guo LW, Sherman ELC, Zinn KR, Welch MJ (1996) *Bioconjug Chem* 7:511–522
- Cutler CS, Wuest M, Anderson CJ, Reichert DE, Sun Y, Martell AE, Welch MJ (2000) *Nucl Med Biol* 27:375–380
- Jones-Wilson TM, Deal KA, Anderson CJ, McCarthy DW, Kovacs Z, Motekaitis RJ, Sherry D, Martell AE, Welch MJ (1998) *Nucl Med Biol* 25:523–530
- Kabachnik MI, Medved' TY, Polikarpov YM, Pasechnik MP (1984) *Izv Akad Nauk SSSR Ser Khim* 844–849
- Kabachnik MI, Medved' TY, Bel'skii FI, Pasechnik MP (1984) *Bull Acad Sci USSR* 33:777
- Bansal N, Germann MJ, Seshan V, Shires GT, Malloy CR, Sherry AD (1993) *Biochemistry* 32:5638–5643
- Burai L, Király R, Lázár I, Brücher E (2001) *Eur J Inorg Chem* 813–820
- Buster DC, Castro MMCA, Geraldès CFGC, Malloy CR, Sherry AD, Siemers TC (1990) *Magn Reson Med* 15:25–32
- Delgado R, Siegfried LC, Kaden TA (1990) *Helv Chim Acta* 73:140–148
- Geraldès CFGC, Sherry AD, Cacheris WP (1989) *Inorg Chem* 28:3336–3341
- Geraldès CFGC, Sherry AD, Lázár I, Misteta A, Bogner P, Berényi E, Sümegi B, Kiefer GE, McMillan K, Maton F, Müller RN (1993) *Magn Reson Med* 30:696–703
- Kim WD, Kiefer GE, Huskens J, Sherry AD (1997) *Inorg Chem* 36:4128–4134
- Lázár I, Hrcir DC, Kim WD, Kiefer GE, Sherry AD (1992) *Inorg Chem* 31:4422–4424
- Shapiro EM, Borthakur A, Bansal N, Leigh JS, Reddy R (2000) *J Magn Reson* 143:213–216

24. Sherry AD, Ren J, Huskens J, Brucker E, Toth E, Geraldes CFGC, Castro MM, Cacheris WP (1996) *Inorg Chem* 35:4604–4612
25. Volkert WA, Hoffmann TJ (1999) *Chem Rev* 99:2269–2292
26. Makos JD, Malloy CR, Sherry AD (1998) *J Appl Physiol* 85:1800–1805
27. Winter PM, Seshan V, Makos JD, Sherry AD, Malloy CR, Bansal N (1998) *J Appl Physiol* 85:1806–1812
28. Hassfjell S, Bruland OS, Hoff P (1997) *Nucl Med Biol* 24:231–237
29. Hassfjell S, Ingebrigtsen K, Bruland OS (2001) *Nucl Med Biol* 28:425–433
30. Kothari K, Samuel G, Banerjee S, Unni PR, Sarma HD, Chaudhari PR, Unnikrishnan TP, Pillai MRA (2001) *Nucl Med Biol* 28:709–717
31. Burai L, Ren J, Kovacs Z, Brücher E, Sherry AD (1998) *Inorg Chem* 37:69–75
32. Lukes I, Kotek J, Vojtisek P, Hermann P (2001) *Coord Chem Rev* 216–217:287–312
33. Kotek J, Hermann P, Cisarova I, Rohovec J, Lukes I (2001) *Inorg Chim Acta* 317:324–330
34. Geraldes CFGC, Marques MP, de Castro B, Pereira E (2000) *Eur J Inorg Chem* 559–565
35. Antipin MY, Baranov AP, Kabachnik MI, Pisreva SR, Polikarpov YM, Sinyasvskaya EI, Struchkov YT (1996) *Heteroatom Chem* 7:229–232
36. Rohovec J, Vojtišek P, Hermann P, Ludvík J, Lukeš I (2000) *J Chem Soc Dalton Trans* 141–148
37. Rohovec J, Vojtišek P, Hermann P, Mosinger J, Žák Z, Lukeš I (1999) *J Chem Soc Dalton Trans* 3585–3592
38. Huskens J, Torres DA, Kovacs Z, André J, Geraldes CFGC, Sherry AD (1997) *Inorg Chem* 36:1495–1503
39. Kovacs Z, Sherry AD (1997) *Synthesis* 759–763
40. Sherry AD, Malloy CR, Jeffrey J, Brucker E, Toth E, Cacheris WP, Geraldes CFGC (1988) *J Magn Reson* 76:528–533
41. Dischino DD, Delaney EJ, Emswiler JE, Gaughan GT, Prasad JS, Srivastava SK, Tweedle MF (1991) *Inorg Chem* 30:1265–1269
42. Martell AE, Motekaitis RJ (1992) *Determination and use of stability constants*. VCH, New York
43. Sherry AD, Cacheris WP, Kuan KT (1988) *Magn Reson Med* 8:180–190
44. Bass LA, Wang M, Welch MJ, Anderson CJ (2000) *Bioconjug Chem* 11:527–532
45. Bass LA, Lanahan MV, Duncan JR, Erion JL, Srinivasan A, Schmidt MA, Anderson CJ (1998) *Bioconjug Chem* 9:192–200
46. Green MA, Welch MJ, Mathias CJ, Fox KA, Knabb RM, Huffman JC (1985) *J Nucl Med* 26:170–180
47. Sun X, Wuest M, Weisman GR, Wong EH, Reed DP, Boswell CA, Motekaitis R, Martell AE, Welch MJ, Anderson CJ (2002) *J Med Chem* 45:469–477
48. Vincent CA (1976) *J Chem Educ* 53:490–493
49. Motekaitis RJ, Rogers BE, Reichert DE, Martell AE, Welch MJ (1996) *Inorg Chem* 35:3821–3827
50. Kumar K, Tweedle MF, Malley MF, Gougoutas JZ (1995) *Inorg Chem* 34:6472–6480
51. Delgado R, Frausto da Silva JJR (1982) *Talanta* 29:815–822
52. Stetter H, Frank W (1976) *Angew Chem Int Ed Engl* 15:686