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The Effect of the Amide Substituent on the Biodistribution and Tolerance of Lanthanide(III) DOTA-Tetraamide Derivatives

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Abstract

Objectives—Recent advances in the design of MRI contrast agents have rendered the lanthanide complexes of DOTA-tetraamide ligands of considerable interest, both as responsive MR agents and paramagnetic chemical exchange saturation transfer agents. The potential utility of these complexes for in vivo applications is contingent upon them being well tolerated by the body. The purpose of this study was to examine how the nature of the amide substituent, and in particular its charge, affected the fate of these chelates postinjection.

Materials and Methods—Complexes of 6 DOTA-tetraamide ligands were prepared in which the nature of the amide substituent was systematically altered. The 6 ligands formed 3 series: a phosphonate series that included tri-cationic, mono-anionic, and polyanionic complexes; a carboxylate series made up of a tri-cationic complex and a mono-anionic complex; and lastly, a tri-cationic complex with an aromatic amide substituent. These complexes were labeled with an appropriate radioisotope, either ¹⁵³Gd or ¹⁷⁷Lu, and the biodistribution profiles in rats recorded 2 hours postinjection.

Results—Biodistribution profiles were initially acquired at low doses to minimize adverse effects. All the complexes studied were found to be excreted primarily through the renal system, with the majority of the dose being found in the urine. None of the complexes exhibited substantial uptake by bone, liver, and spleen, except for a complex with 4 phosphonate groups that exhibited significant bone targeting capabilities. Increasing the dose of each complex to that of a typical MR contrast agent

was found to render all 3 tri-cationic complexes studied here acutely toxic. In contrast, no ill effects were observed after administration of similar doses of the corresponding anionic complexes.

Conclusions—The absence of uptake by the liver and spleen indicate that irrespective of the ligand structure and charge, these complexes are not prone to dissociation in vivo. This is in agreement with previously published work that indicates high kinetic inertness for this class of compounds. At low doses, all complexes were well tolerated; however, for applications that require higher doses, the structure and charge of the ligand becomes a fundamentally important parameter. The results reported herein demonstrate the importance of incorporating negatively charged groups on amide substituents if a DOTA-tetraamide complex is to be employed at high doses in vivo.

Keywords

Lanthanide complexes; Biodistribution; MRI contrast agents; PARACEST agents

Complexes of Gd^{3+} have found widespread application as contrast agents in magnetic resonance imaging (MRI)^{1,2} in which they are administered in relatively high doses, typically 0.1–0.5 mmol kg⁻¹. Numerous Gd^{3+} complexes have been approved for clinical use and, without exception, the ligand systems employed are octadentate polyamino-polycarboxylates that render the resulting complex either neutral or negatively charged.^{1,2} These complexes have high thermodynamic stability constants and are considered inert to dissociation under physiological conditions³; they are therefore excreted intact, usually through the renal system,^{3,4} within several hours of injection. All clinically approved agents also have in common a single coordinated water molecule. This water molecule, which is in relatively rapid exchange with the bulk solvent, contributes significantly to the relaxivity of the contrast agent. Relaxivity, the increase in water proton relaxation rate per unit concentration of contrast agent, is the measure of the effectiveness of the agent. One of the most successful contrast agents is the Gd^{3+} complex of DOTA (H_4DOTA = 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid), a ligand based on the tetraazamacrocyclic cyclen.

The tetraamide derivatives of DOTA (generic structure shown in Fig. 1) form complexes with lanthanide ions that are structurally analogous to those of DOTA.⁵ However, unless ionizable groups, such as carboxylates or phosphonates, are incorporated into the ligand, the resulting Ln^{3+} complexes will be tri-cationic in nature. It is now an accepted feature of these complexes that the exchange kinetics of the coordinated water molecule is much slower than that in the corresponding polyamino-polycarboxylate complexes.^{5–9} The slow water exchange kinetics of these DOTA-tetraamide complexes was found to limit their relaxivities,^{5–9} and hence their potential as MRI contrast agents. However, this perceived shortcoming has since been found to be a favorable attribute. The Gd^{3+} complex of a DOTA-tetraamide ligand that has methylene phosphonate amide substituents, **Gd1** (see Appendix), was found to act as a pH-sensing contrast agent.¹⁰ The mechanism of action of this agent involves the pH-dependent acceleration of water proton exchange between the coordinated and bulk water by the pendant phosphonate groups.¹⁰ This gives rise to low relaxivity when exchange is slow and higher relaxivity under the faster exchange regimen. The pH range over which this complex exhibits its change in relaxivity is ideal for measuring change in pH in vivo and pH maps of kidneys and tumors in mice have been acquired using this agent.^{11–13}

DOTA-tetraamide complexes have also shown the potential for generating contrast in MR imaging through chemical exchange saturation transfer (CEST). These paramagnetic CEST agents, often referred to as PARACEST agents,^{14,15} offer some potential advantages over conventional Gd^{3+} -based contrast agents. In the complexes of Ln^{3+} ions that induce large hyperfine shifts, such as Eu^{3+} , Tm^{3+} , Dy^{3+} and Nd^{3+} , the protons of the slowly exchanging coordinated water molecule are shifted far away from the bulk water and hence can easily be saturated by use of a frequency-selective presaturation pulse without directly saturating the

solvent water signal itself. Subsequent exchange of these protons with those of the bulk transfers the saturation to the solvent water, thereby reducing its signal intensity and resulting in negative image contrast.

The CEST properties of an agent are assessed by recording a CEST spectrum in which the solvent water signal intensity, as a percentage of its initial intensity (M_S/M_0 %), is plotted against the frequency offset of the presaturation pulse.¹⁴ Eu^{3+} is commonly employed as the basis of PARACEST agents because it has been reported to have the slowest water exchange kinetics of all Ln^{3+} ions in this type of complex.¹⁶ The CEST spectra of **Eu2** show a peak at $\sim +50$ ppm characteristic of CEST arising from the water molecule coordinated to Eu^{3+} (Fig. 2). The more intense peak at 0 ppm is the result of direct saturation of the solvent water protons. The Ln^{3+} complexes of **4** and **5** have also been extensively studied and found to have favorable CEST properties.^{9,17–23} In contrast, the exchange kinetics of the coordinated water molecule protons in **Ln1** complexes are too rapid for a CEST effect to be observed for **Eu1**. Fitting the CEST spectra of **Eu2** to the Bloch equations modified for exchange²⁴ afforded a value of $\tau_M = 67 \pm 6 \mu\text{s}$ ($\tau_M = 1/k_{\text{ex}}$) longer than that of **Ln1** but shorter than those of **Eu3** ($\tau_M = 1.3$ milliseconds).²⁵

This new method of generating image contrast offers several advantages over conventional agents, particularly in the development of targeted agents (in which motion artifacts can be reduced by interleaving images acquired with and without saturation)²⁶ and responsive agents (in which ratio-metric detection can be used to eliminate the question of contrast agent concentration).²⁷ However, if this class of compounds is to be used for in vivo applications then developing an understanding of how these compounds are tolerated by the body and their fate postinjection is imperative. As a first step toward this understanding, we have undertaken some in vivo investigations of a number of DOTA-tetraamide complexes (see Appendix) that differ in overall charge (see structures in Fig. 1).

MATERIALS AND METHODS

General Remarks

All solvents and reagents were purchased from commercial sources and used as received unless otherwise stated. The preparation of ligands **1**,¹⁰ **3**,¹⁰ **4**,²⁸ **5**,⁹ and **6**⁸ has been described previously. ^1H and ^{13}C NMR spectra were recorded on a Bruker Avance III spectrometer operating at 400.13 and 100.62 MHz, respectively. ^1H NMR and CEST spectra were recorded on a Varian Mercury spectrometer operating at 299.99 MHz.

Synthesis

1,4,7,10-Tetraazacyclododecane-1,4,7,10-Tetraacetamidomethylene-(ethyl) phosphonate Sodium Salt (2)—A 1 M solution of sodium hydroxide (0.4 mL) was added to a solution of ligand **3** (0.1 g, 0.1 mmol) in water (6 mL). The resulting solution was heated, with stirring, at 80°C for 18 hours. After cooling to room temperature, the solvents were removed by freeze-drying to afford the title compound as a colorless solid in quantitative yield.

Mp, decomposes $>244^\circ\text{C}$; ^1H NMR (400 MHz, D_2O) δ = 3.87 (4H, q, $^3J_{\text{H-H}} = 6$ Hz, OCH_2), 3.85 (4H, q, $^3J_{\text{H-H}} = 6$ Hz, OCH_2), 3.42 (8H, d, $^2J_{\text{P-H}} = 15$ Hz, PCH_2NH), 3.13 (8H, s br, NCH_2CO), 2.59 (8H, s br, ring NCH_2), 2.38 (8H, s br, ring NCH_2), 1.18 (12H, t, $^3J_{\text{H-H}} = 6$ Hz, CH_3); ^{13}C NMR (100 MHz, D_2O) δ = 16.1 (d, $3J_{\text{P-C}} = 6$ Hz, CH_3), 35.6 (d, $1J_{\text{P-C}} = 147$ Hz, PCH_2NH), 50.2 (s br, ring NCH_2), 57.0 (s, NCH_2CO), 61.3 (d, $2J_{\text{P-C}} = 6$ Hz, OCH_2), 172.9 (d, $3J_{\text{P-C}} = 6$ Hz, $\text{C}=\text{O}$); ^{31}P NMR (121 MHz, D_2O) δ = 19.1 (s, $\text{CH}_2\text{P}(\text{OEt})\text{O}_2\text{Na}$); m/z (ESI $-$) = 887 (60%, $[\text{H}_3\text{L}]^-$), 909 (100%, $[\text{NaH}_2\text{L}]^-$), 925 (75%, $[\text{KH}_2\text{L}]^-$) 931 (90%, $[\text{Na}_2\text{HL}]^-$),

947 (90%, [NaKHL][−]), 953 (70%, [Na₃L][−]), 969 (45%, [Na₂KL][−]); Anal found C 26.8%, H 6.1%, N 8.7%; C₂₈H₅₆N₈Na₄O₁₆P₄ • KCl • 11H₂O requires C 26.9%, H 6.3%, N 9.0%.

Europium(III) 1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetamidomethylene-(ethyl)phosphonate Sodium Salt (Eu2)—Ligand **2** (50 mg, 51 μmol) was dissolved in water (5 mL) and europium chloride (17 mg, 46 μmol) added. The solution was heated, with stirring, to 60°C for 18 hours. The solvents were then lyophilized to afford the title complex as a colorless solid in quantitative yield. The complex was used without further purification.

¹H NMR (300 MHz, D₂O) δ = 24.88 (4H, s, ring *ax*^S), 3.16 (4H, s, CH₂P), 0.83 (4H, s, CH₂P), −2.89 (4H, s, ring *eq*^S), −5.02 (4H, s, ring *ax*^C), −8.56 (4H, s, ring *eq*^C), −9.96 (4H, s, *ac*), −12.86 (4H, s, *ac*).

Radiochemistry

¹⁷⁷Lu Method 1—¹⁷⁷Lu, produced by neutron irradiation of isotopically enriched ¹⁷⁶Lu (NO₃)₃ and dissolved in 0.05N HCl, was purchased from the University Missouri Research Reactor. All reaction vials were washed with 10%–20% nitric acid overnight. Ligands **2**, **3**, and **5** were labeled with ¹⁷⁷Lu by adding 1 μL of the ¹⁷⁷LuCl₃ solution (100–200 μCi) to 100 μL of a 0.4 M ammonium acetate buffer (pH = 7.5) containing the ligand solution at 5 mM. The reaction mixture was then incubated at 90°C for 1 hour in an Eppendorf thermo-mixer at 1,000 rpm. The radiochemical reactions were monitored by a Rita Star Radioisotope TLC Analyzer (Straubenhardt, Germany).

¹⁷⁷Lu Method 2—A stock isotope solution was prepared by adding 2 μL of a 0.3 mM solution of ¹⁷⁷LuCl₃ in 0.1 M HCl to a 3 × 10^{−4} M LuCl₃ carrier solution. Appropriate ligand solutions were then prepared at approximately the same concentration in deionized water. Complexation was conducted by combining a 5% excess of a ligand solution with the isotope (pH 2) and gradually increasing the pH to 7 by addition of 0.1 M NaOH. Complexation was monitored and the final complex purified by passing the solution (100 μL) through a Sephadex C-25 column eluting with 4:1 saline (0.85% NaCl/NH₄OH; 2 × 3 mL). The amount of radioactivity in the eluent (complex) *versus* the amount remaining on the column (free metal) provided an index of complexation.

¹⁵³Sm Method—¹⁵³Sm₂O₃, with a specific activity >5 GBq/mg, was produced at the ITN (Instituto Tecnológico e Nuclear), Lisbon. The ¹⁵³Sm-oxide was prepared from a 98% enriched target, sealed into a quartz vial and welded into an aluminum can, by neutron irradiation using a thermal flux of 2.3 × 10¹³ n/cm² s. Following irradiation, the sample was opened, dissolved in 1N HCl and brought to a stock concentration of 1.9 × 10^{−3} M. Stock solutions of the ligand **6** (30 μg) were prepared in isotonic HEPES pH 6.8 buffer (2 mL), mixed with the radioactive metal chloride (4 mCi), heated for 10 hours at 85°C, and kept overnight at room temperature. The radiochemical purity of the ¹⁵³Sm**6** solutions was determined by thin layer chromatography, and the percentage of bound metal averaged 96%.

¹⁵³Gd Method—The concentration of ligand **1** was estimated by dissolving a known amount in D₂O and comparing proton integrals to those of a standard, potassium hydrogen phthalate. Ligand **1** (151.2 mg) was dissolved in 2 mL of nanopure water and 241 μCi of ¹⁵³GdCl₃ (Perkin Elmer Life Sciences, Billerica MA) was added. The pH was adjusted to 4 and the solution was heated at 60°C overnight. Solid GdCl₃ (60.31 mg, 229 μmol) was added to the solution, the pH adjusted to 5.5 with 1N sodium hydroxide and the solution stirred at 60°C for a week. Radio-high-performance liquid chromatography (HPLC) indicated 3 peaks: free ¹⁵³Gd, labeled Gd**1**, and a later eluting impurity. The solution was purified using a 500 mg C-18 SepPak that

had been pre-equilibrated with ethanol and then water. The radio-labeled mixture was put on the column and eluted first with water and then a 3:1 water: ethanol mixture to elute the Gd1. Fractions were analyzed by HPLC and pure fractions combined. Solvent was removed under vacuum and the solid was reconstituted in water for the biodistribution studies. Concentration of cold Gd1 was estimated from the activity in the sample of known volume.

GdDTPA was labeled with ^{111}In by an exchange reaction. A gadopentetate dimeglumine (Magnevist, Berlex, Wayne NJ) was diluted to 20 mM and to a 0.5 mL aliquot of this solution was added $^{111}\text{InCl}_3$ (Perkin Elmer Life Sciences, Billerica MA) and the pH adjusted to 4. The solution was stirred at 40°C for 1 hour and the pH adjusted to 7 with sodium hydroxide. The radiochemical purity was assessed by TLC and was >99%.

Biodistribution Studies

All animal studies were performed in compliance with guidelines set by the Institutional Animal Care and Use Committee (IACUC) of the University of Texas Southwestern Medical Center at Dallas and the IACUC at Epix Pharmaceuticals. The ^{177}Lu -labeled chelates were diluted with 10 mM PBS (pH 7.4). Normal male Sprague-Dawley rats (Harlan, IN) weighing 170–220 g were injected with 5 μCi of the ^{177}Lu -labeled ligand *via* the tail vein. The total injected volume was 100 μL . The rats were anesthetized before being killed 2 hours postinjection ($n = 4$). 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). Total percentage uptake in the bone was calculated by assuming that the femur represents 1/25 of the total skeletal mass with a uniform distribution. Representative samples of muscle and blood were counted and the total percentage uptake calculated by assuming a total mass of 43% and 6.5%, respectively.²⁹

Conscious male Sprague-Dawley rats (Charles River, Wilmington MA), 325–350 g, were injected with 7 μCi $^{153}\text{Gd1}$ (35 $\mu\text{mol kg}^{-1}$ Gd1) and 10 μCi of $^{111}\text{InDTPA}$ (68 $\mu\text{mol kg}^{-1}$ GdDTPA) *via* the tail vein. The labeled compounds were mixed immediately before injection. The total injection volume was 250 μL . Blood samples were taken from the carotid artery, which had been cannulated with a silicone tube before the study. Blood (150–200 μL) was taken before, and 1, 3, 5, 10, 15, 30, 60, 90, and 120 minutes postinjection of the labeled compounds. At 120 minutes, the animals ($n = 3$) were euthanized by pentobarbital overdose and the organs of interest were removed, weighed, and counted. Aliquots of the injection solution were also weighed and counted to calculate %ID/g and %ID/organ. Three more animals were injected *via* the tail vein, but without blood collection. These rats were killed 7 days after injection, and their organs removed, weighed, and counted along with aliquots of the injection solution.

Determination of Partition Coefficients

The partition coefficients ($\log P$) of $^{177}\text{Lu2}$ and $^{177}\text{Lu3}$ were determined by adding 5 μL of a 5 mM solution of each complex to a biphasic solution containing 500 μL of octanol and 500 μL of water (obtained from presaturated octanol/water, $n = 10$). The resulting mixture was shaken at room temperature for 1 hour and then allowed to settle. From each of the 10 samples, 100 μL aliquots of the water and octanol phases were removed. The partition coefficient was calculated as a ratio of the counts in the octanol fraction to the counts in the water fraction. An average $\log P$ value was obtained from the 10 samples.

γ -Imaging

A gamma camera-computer system (GE 400 Genie-Acq, General Electrics, Milwaukee) was used for acquisition and preprocessing. Data processing and display were performed on a personal computer using homemade software developed for the IDL 5.2 computer tool. A well-

counter (DPC-Gamma C₁₂; LA) with a Compaq DeskPro compatible computer was used for activity counting in the biodistribution studies. Dynamic gamma images were obtained using 250 g Wistar rats. All animal studies were carried out in compliance with procedures approved by the appropriate institutional review committees. Conscious rats were allowed free access of food and water ad libitum. Wistar rats (groups of 4 animals) were injected in the tail vein with ca. 500 μ Ci of the ¹⁵³Sm6 chelate. The animals were positioned in ventral de-cubitus over the detector. Image acquisition was started immediately before the injection, using 64 $\times$ 64 matrices, the total acquisition time was 30 minutes. Images were subsequently processed using an IDL-based program (Interactive Data Language, Research Systems, Boulder, CO). To analyze the transport of radiotracer over time, 4 regions of interest were drawn on the image files, corresponding to the brain, thorax, liver, and left kidney and time-activity curves were obtained.

RESULTS

Biodistribution Studies

¹⁵³Gd1 and the ¹⁷⁷Lu complexes of ligands 2 to 6 were administered to rats *via* tail vein injection. Having been previously administered to mice with no adverse effects^{11,12} a relatively high dose of ¹⁵³Gd1 was administered: $\sim 35 \mu\text{mol kg}^{-1}$. All other complexes were administered at lower doses (1.2–1.5 $\mu\text{mol kg}^{-1}$) to minimize possible adverse effects. In all cases, no adverse reactions to the administration of the complexes were observed. The urine was collected and at 2 hours postinjection the rats were killed, the organs excised, and the radioactivity of each organ and urine was assessed. In each case, >95% of the total injected radioactive dose was recovered. The data obtained are presented as % ID/g in Table 1 and % ID/organ in Table 2. Two hours postinjection, the majority (>90% of the injected dose) of each complex was found in the animals' urine (data not shown).

Biodistribution studies at a typical MRI dose of 0.1 mmol kg⁻¹ using ¹⁷⁷Lu5 and ¹⁷⁷Lu6 were attempted. In each case, the animals were found to expire within 1 minute of administration of the tri-cationic complex. As a result biodistribution studies at this higher dose were not completed. Significantly, animals administered with the same 0.1 mmol kg⁻¹ dose of Gd1 or Eu4 exhibited no adverse effects.

Pharmacokinetics of Gd1

The blood clearance of Gd1 was simultaneously compared with that of a similar hydrophilic extracellular tracer, such as GdDTPA, to provide an assessment of its blood clearance kinetics. There is little overlap between the γ -emission range of ¹¹¹In and ¹⁵³Gd and so both these isotopes can be measured simultaneously. ¹¹¹In was substituted for ¹⁵³Gd in the DTPA complex and ¹⁵³Gd1 and ¹¹¹InDTPA were administered to rats simultaneously to minimize interanimal variability. After administration of the 2 complexes, blood was sampled at 9 time-points from 1 to 120 minutes postinjection. Both compounds exhibited bi-exponential blood clearance (Fig. 3), and a 2 compartment model was used to fit the data; the relevant fitting parameters are listed in Table 3. Despite interanimal variation, the ratio of clearance rates for Gd1 versus InDTPA, within a given animal, were very similar. Significantly, the blood half-life of Gd1 was found to be 60% shorter than that of InDTPA, which may be related to its fast, and possibly irreversible, uptake in the bone. The bone uptake of ¹⁵³Gd1 is highlighted by the 2-hour biodistribution of coinjected ¹⁵³Gd1 and ¹¹¹InDTPA, which reveals substantially higher levels of bone activity for ¹⁵³Gd1.

γ -Imaging Studies

A convenient method for following the biodistribution of a complex immediately after injection is γ -imaging. By administering the complex radiolabeled with ¹⁵³Sm, a γ -emitter, images can

be obtained using a simple γ -camera. Although this technique affords only qualitative information about the distribution of the complex, it has the advantage that images can be taken at rapid time intervals postadministration and the animals need not be killed. Figure 4 shows images of a rat administered with a $0.2 \mu\text{mol kg}^{-1}$ dose of $^{153}\text{Sm6}$ at 3 time-points postinjection. In the first image, taken 1 minute postinjection, the greatest signal intensity arises from organs in the thorax: heart and lungs. Weaker, but clearly distinguishable, signals are also observed from the kidneys. Images taken at 5 and 9 minutes postinjection clearly show the complex being removed from the blood-pool by the kidneys and excreted into the urine and bladder. A composite of all the images taken over this time period (Fig. 4 bottom) provides a clear picture of which organs are exposed to the complex in the minutes postinjection: the heart, kidneys, bladder, and urethra can all be identified.

Mean activity/pixel values were obtained for 30 minutes after the injection of $^{153}\text{Sm6}$ into a different animal. Three regions of interest (ROIs) were identified: 1 that included parts of the liver and spleen; one for the thorax, containing primarily the heart and lungs; and 1 covering part of the kidney. For each ROI, the curves were normalized relative to the maximum activity observed for any pixel and then smoothed. The curves (Fig. 5) show a rapid increase in activity as the complex is administered (<1 min) followed by continued activity over the course of the experiment. Owing to the nature of the acquisition technique absolute intensities cannot be compared in these curves, with differences in the tissue depth giving rise to different activities in each ROI. However, the persistence of activity in the thorax indicates that a substantial amount of the complex is retained by the blood showing up in the heart and lungs, both of which are well perfused. This observation is consistent with the known affinity of this complex for serum albumins.⁸ The complex is observed to slowly eliminate through the kidneys; this slow elimination is also probably a product of the strong retention of the complex by the blood.

Perfused Heart Studies

The effect of a tri-cationic complex upon heart tissue was examined in an isolated perfused heart model. Hearts from anesthetized rats were surgically removed and immediately attached *via* the aorta to a Langendorff perfusion apparatus and perfused with 200 mL of a bicarbonate Krebs-Henseleit (KH) buffer (pH 7.4). The developed pressure, an indicator of heart function, was monitored as Eu5 was added to the perfusate. Aliquots of $80 \mu\text{mol}$ of Eu5 were added to the perfusate at 10-minute intervals for 40 minutes. The dosing was then stepped up to $160 \mu\text{mol}$ per addition and a further 4 aliquots added at 10-minute intervals. After each addition, an immediate drop in the developed pressure was observed. After the first addition of Eu5, the developed pressure recovered to a value higher than the initial value, but each subsequent addition of Eu5 resulted in a decrease in the developed pressure once the heart stabilized (Fig. 6). Twenty minutes after the last addition of Eu5, the perfusate was replaced with fresh KH buffer free of Eu5 and the developed pressure and heart rate quickly recovered to near initial values (final heart rate = 255 bpm *c.f.* initial heart rate = 250 bpm).

DISCUSSION

Ligands **1–6** afford an opportunity to study the effect of the overall charge a complex has on its properties. The Ln^{3+} complexes of ligands **3**, **5**, and **6** complexes, all of which are neutral ligand systems, are tri-cationic. The overall charge of the complex can be altered by incorporating ionisable groups into the amide substituents. Incorporating anionic amide substituents affords mono-anionic complexes. Carboxylic acids and phosphonate mono-esters generally have low protonation constants³⁰ and remain deprotonated (anionic) over the physiologically relevant pH range (pH 4 – 8); in consequence complexes **Ln2** and **Ln4** are mono-anionic. The situation is more complex in the case of **Ln1** complexes. Each phosphonate has two protonation constants: one low and one in the pH range 5– 8 region,³¹ which means

that there is a distribution of protonation states over this pH range. However, from a previously published speciation diagram of these protonation states, it can be determined that at physiological pH, around 60% of the Ln1 complex is completely deprotonated, ie, penta-anionic, and 40% is mono-protonated and thus tetra-anionic.¹⁰ Of particular interest here was the effect of this change in charge upon the in vivo tolerance and biodistribution of these complexes. Clearly, if these complexes are to be used for MR imaging purposes, and at the high doses that MR imaging implies, it is important that they are well tolerated in vivo. Gd1 has been used in vivo to successfully acquire pH maps of mice without apparent adverse side effects.^{11–13} On the other hand, anecdotal evidence has suggested that some tri-cationic lanthanide DOTA-tetraamide derivatives may be toxic to rodents, and perhaps mammals in general.

Our own interest in these Ln³⁺ DOTA-tetraamide complexes is their potential application as MRI contrast agents, although these complexes may also be potentially useful in the field of nuclear medicine. As pH responsive agents,^{10,12,13} interest centers around Gd³⁺ but as PARACEST agents,¹⁴ any other paramagnetic Ln³⁺ ion may be of interest. Fortunately, all Ln³⁺ ions exhibit very similar coordination chemistry and with the DOTA-tetraamide ligands studied here, they all adopt the same mono-capped square antiprismatic coordination geometry.^{8–10} This means that the choice of Ln³⁺ ion is somewhat arbitrary since differences in the in vivo behavior resulting from changing the metal ion are likely to be smaller than the errors in our measurements. Because of our interest in its pH responsive behavior, ¹⁵³Gd was chosen as a radiolabel for ligand 1. For all other ligands, ¹⁷⁷Lu was chosen because of convenient access to this isotope.

It is extremely important that a complex proposed for in vivo application is resistant to dissociation under physiological conditions. Both ligands and Ln³⁺ ions are toxic (DOTA: LD₅₀ = 0.18 mmol kg⁻¹;³² GdCl₃: LD₅₀ = 0.35 mmol kg⁻¹;³³ i.v. in mice), so at high doses a complex that rapidly releases its metal ion would be toxic. Although the thermodynamic stability constants determined for a range of lanthanide DOTA-tetraamide complexes are as much as 6 orders of magnitude lower than the corresponding DOTA complexes,^{28,34} critically this type of complex exhibits dissociation kinetics that are as much as 3 or 4 orders of magnitude slower than the corresponding complexes of DOTA.^{28,34} This means that if DOTA-tetraamide complexes are eliminated from the body as quickly as those of DOTA, then it is reasonable to expect that in vivo dissociation will not occur to any significant extent. From the point of view of complex dissociation, this *should* render these complexes safe for use in vivo.

Biodistribution studies can provide a valuable insight into the fate of a complex in vivo. The free Ln³⁺ ion is a surrogate for the Ca²⁺ ion and consequently accumulates in teeth and bone^{4,35,36}; it may also form colloids with serum proteins that are cleared through the reticuloendothelial system (RES) and Kupffer cells giving rise to higher than normal levels of activity in the liver and spleen.^{4,35,36} Radioactivity in these organs can be an indication of complex dissociation in vivo.

Anionic LnDOTA-Tetraamide Complexes

All 3 anionic DOTA-tetraamide complexes studied here, Ln1, Ln2, and Ln4, were found to be excreted rapidly and exclusively through the renal system. At the low doses required for nuclear medicine applications (1–35 μmol kg⁻¹), these anionic complexes were well tolerated by the animals. At the relatively high doses required for conventional MRI contrast agents (≥0.1 mmol kg⁻¹), complexes Ln1 and Ln4 were also found to be well tolerated in vivo and no adverse effects were noted. Ln2 was not studied at higher doses. The kinetic inertness of the LnDOTA-tetraamide complexes^{28,34} seems to render these complexes stable enough for use in vivo, and the biodistribution data is consistent with the complexes remaining intact in vivo. In all cases, the levels of activity in the liver and spleen were low for each complex. The level of

activity in bone, another key indicator of complex dissociation, was also low for Ln2 and Ln4. Indeed, the low levels of residual activity in all organs observed for these 2 mono-anionic complexes 2 hours postinjection is reminiscent of the biodistribution profiles observed for clinically approved macrocyclic contrast agents.^{3,4,37} Both Ln2 and Ln4 exhibit favorable CEST profiles, even if their water exchange kinetics are faster than optimal as predicted by theory.^{14,24} Since PARACEST agents are currently not yet as sensitive as conventional MRI contrast agents, it is not yet clear what an effective dose of these agents would be. However, the results of these studies are encouraging suggesting that, despite their relatively low thermodynamic stabilities,^{28,34} these mono-anionic DOTA-tetraamide complexes are well tolerated in vivo and are potentially viable as contrast media.

The level of activity in bone was higher in the case of Ln1 although this can be attributed to the known affinity of phosphonate ligands for bone.^{38–42} This means it is likely that Ln1 itself binds to bone rather than dissociating to free Ln³⁺ before bone uptake. The absence of elevated levels of activity in the liver and spleen for Ln1, hallmarks of a free Ln³⁺ ion, are consistent with the complex remaining intact. Ln1 is cleared from the blood more rapidly than complexes of DTPA. This is probably the result of rapid kidney uptake and excretion coupled with rapid uptake in the bone. The biodistribution data expressed as percent injected dose/organ (Table 2) clearly shows the marked preference of ¹⁵³Gd1 for bone, confirming the targeting effect of the phosphonate groups. When ¹⁵³Gd1 was administered to another cohort of rats and its biodistribution determined 7 days postinjection, the level of ¹⁵³Gd1 in bone was almost identical to the 2 hour values. No retention of this complex in any of the other organs was observed 7 days postinjection. The clear bone targeting properties of Ln1 may render it useful for nuclear medicine applications in which bone targeting is desirable. It is a less attractive property in an MRI contrast agent; the higher doses involved and the persistence of the complex in bone raises certain questions over the long-term safety of administering high doses of this complex. Notably, the apparent bone targeting effect of the phosphonates can be masked by inclusion of at least one ester function in each phosphonate group; neither ¹⁷⁷Lu2 nor ¹⁷⁷Lu3 exhibit the same preference for bone. This effect seems to be analogous to that observed when a phosphonate is replaced by a methyl or benzyl phosphinate, which has also been shown to eliminate bone targeting effects.⁴³ So although the polyanionic Gd1 certainly has attractive pH reporting properties and is initially well tolerated in vivo, the long-term implications of, possibly irreversible, bone uptake would need to be carefully examined before this complex could be considered as an MRI contrast agent for clinical applications.

Cationic LnDOTA-Tetraamide Complexes

At low doses, all 3 cationic complexes studied here, Ln3, Ln5, and Ln6, were well tolerated when administered intravenously by tail-vein injection. The biodistribution profiles show that each of these complexes is primarily excreted through the renal system, and like the anionic complexes, excretion is rapid. In vitro these cationic LnDOTA-tetra-amide complexes are as kinetically inert to dissociation as their anionic counterparts,^{28,34} so it is likely that they are sufficiently inert to avoid dissociation in vivo. However, although the biodistribution data acquired for the 3 cationic complexes studied here do not suggest any gross dissociation of the complexes, it is noteworthy that the level of activity recorded in organs associated with free Ln³⁺ ion uptake (bone, liver, and spleen) is slightly higher for the cationic complexes than that for the anionic complexes, the bone uptake of Gd1 being the notable exception. The increased activity in the liver observed for ¹⁷⁷Lu3 relative to that of ¹⁷⁷Lu2 does not seem to be the result of an increase in hydrophobicity arising from the presence of a second ethyl group per phosphonate. The 2 complexes have very similar and negative log *P* values: log *P* = -3.77 ± 0.37 (¹⁷⁷Lu2) and log *P* = -3.37 ± 0.19 (¹⁷⁷Lu3). Complexes with large negative log *P* values usually require organic targeting groups, such as the aromatic substituents of GdEOB-DTPA and GdBOPTA, to induce liver uptake through hepatocytes.^{44–46} Since such groups are absent

from $^{177}\text{Lu}3$ and $^{177}\text{Lu}4$, they would not normally be expected to clear through the liver, although the aromatic groups of $^{177}\text{Lu}6$ may induce some hepatobiliary clearance.^{44–46} There are 2 possible explanations for the elevated activity levels observed in the spleen and liver for tri-cationic complexes: the least likely is that a small quantity of complex dissociates in vivo and that the Ln^{3+} ion is liberated. The absence of significant bone uptake at later time points would allow complex dissociation to be definitively ruled out.^{35,36} However, it seems more likely that the cationic complex remains intact, but as a result of its charge, it exhibits behavior that parallels that of the free ion, forming colloids that are subsequently taken up by the liver and spleen. This behavior is particularly evident in the case of $\text{Ln}6$, which is known to associate with serum proteins.⁸ The idea that the tri-cationic DOTA-tetraamide complexes exhibit in vivo behavior that parallels that of the free Ln^{3+} ion is supported by the observed toxicity of these cationic complexes at higher doses.

Complexes $\text{Ln}5$ and $\text{Ln}6$, when administered to rodents by tail vein injection at a typical MRI dosage (0.1 mmol kg^{-1}), were found to be acutely toxic. Death was rapid, within 1 minute of injection, much too rapid to have been¹ caused by dissociation of the Ln^{3+} ion from the chelate cage, which is known to be slow.³⁴ The perfused heart study with $\text{Ln}5$ suggests clearly shows that the complex itself has a negative impact on heart function and presumably a high enough dose will stop heart function altogether. Although mono-cationic CT agents have been found to be relatively toxic,^{47–49} there are some differences in the observed behavior of these 2 classes of compound. The onset of death after administration of tri-cationic DOTA-tetraamide complexes appears to be more rapid than after administration of mono-cationic CT agents. Furthermore, the CT agents were observed to cause motor paresis and cessation of breathing before death.⁴⁸ Cardiac function was not impaired, leading to the suggestion that CT agents had high neural toxicity.⁴⁸ Tri-cationic DOTA-tetraamide complexes did not seem to cause cessation of breathing before death, which seems to indicate that the origin of toxicity in these 2 cationic systems is quite different.

A dose of 0.1 mmol kg^{-1} would afford an average blood concentration level in the region of 1.4 mM. From Figure 6, it can be seen that this amount of $\text{Ln}5$ in the KH perfusion buffer had little negative impact on heart function. However, in this study, rats were administered a bolus injection into the tail vein. The tail vein joins the inferior vena cava, which takes the bolus of the complex directly to the right heart. From there the complex is pumped to the lungs and back to the left heart. This means that immediately postinjection, the heart is exposed to a bolus of the complex at very high concentration.⁵⁰ This effect is clearly visible in the first γ -images of a rat after administration of $\text{Ln}6$ (Fig. 4, top right). This exposure of heart tissue to high concentrations of a tri-cationic complex in the immediate aftermath of a tail vein injection is likely to compromise heart function to such an extent that the onset of death postinjection is rapid. The nature of the interaction between heart tissue and $\text{Eu}5$ remains to be determined but most likely involves the interference of the intact complex with the function of ion channels of the myocytes.

Like cationic CT agents,^{47–49} tri-cationic DOTA-tetra-amide complexes are clearly toxic when used at higher doses. An alternative method of administration, perhaps *via* intra-peritoneal injection or a slower infusion of the agent into the body, may be able to reduce the acute toxicity observed with the cationic complexes $\text{Ln}5$ and $\text{Ln}6$. The toxicity of Ln^{3+} ions is well known to be highly dependent upon the method of administration.³⁵ However, the *i.v.* toxicity observed in these experiments clearly precludes the use of these cationic DOTA-tetraamide complexes in applications requiring the *i.v.* administration of higher effective doses, such as MRI contrast agents. It has been previously reported that negatively charged macrocyclic contrast agents are well tolerated by the cardiovascular system.^{51,52} Our experiments with anionic DOTA-tetraamide complexes indicate that one way in which the

problem of complex toxicity can be overcome by the use of negatively charged ligand systems such as **2** or **4**.

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APPENDIX 1

Ligand **1** = 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetamidomethylenephosphonate; **2** = 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetamidomethylene-(ethyl) phosphonate; **3** = 1,4, 7,10-tetraazacyclododecane-1,4,7,10-tetraacetamidomethylene-(diethyl) phosphonate; **4** = 1,4,7,10-tetraazacyclododecane-1,4, 7,10-tetraacetamidomethylenecarboxylate; **5** = 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetamidomethylene-(ethyl)carboxylate; and **6** = 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetamidomethylene-2-(3-hydroxypyridine).

FIGURE 1.

The structure of some DOTA-tetraamide complexes. “Ln” is a generic symbol for a lanthanide ion.

FIGURE 2.

The CEST spectrum of a 50 mM solution of Eu2 recorded at 7 T, pH 7, 23°C, and $B_1 = 450$ Hz, 320 Hz, 230 Hz, 160 Hz and 110 Hz, irr. time = 2 s. The solid lines are fits to the experimental data.

FIGURE 3. The blood clearance of coinjected $^{153}\text{Gd1}$ (open diamond, dotted line) and $^{111}\text{InDTPA}$ (filled circles, dashed line) demonstrating the more rapid clearance kinetics of Gd1 .

FIGURE 4.

γ -Images of a rat administered with $^{153}\text{Sm6}$. Sixty-second images were taken at 1 minute (upper left), 5 minutes (upper middle), and 9 minutes (upper right) postinjection. A composite γ -image of all 9 frames from the sequential 60-second acquisitions (below).

FIGURE 5.

Averaged time-activity curves for the liver-spleen (light grey), thorax (black), kidney (dark grey) obtained from the γ -images of $^{153}\text{Sm6}$. The curves were normalized relative to the maximum activity obtained from dynamic acquisitions.

FIGURE 6.
The effect of adding Eu5 to a perfused rat heart on the developed pressure of the heart.

TABLE 1
The Biodistribution of Lanthanide DOTA-Tetraamide Complexes 2 h Postinjection Expressed as % Injected Dose/g

	$^{153}\text{Gd}^{3+}$	$^{177}\text{Lu}^{2+}$	$^{177}\text{Lu}^{3+}$	$^{177}\text{Lu}^{4+}$	$^{177}\text{Lu}^{5+}$	$^{177}\text{Lu}^{6+}$
Blood	0.062 ± 0.036	0.005 ± 0.002	0.040 ± 0.008	0.020 ± 0.005	0.039 ± 0.007	0.071 ± 0.024
Heart	0.026 ± 0.011	0.008 ± 0.003	0.029 ± 0.026	0.007 ± 0.003	0.049 ± 0.007	0.048 ± 0.010
Lung	0.078 ± 0.048	0.019 ± 0.005	0.085 ± 0.031	0.021 ± 0.002	0.026 ± 0.011	0.135 ± 0.028
Liver	0.033 ± 0.013	0.037 ± 0.008	0.108 ± 0.023	0.043 ± 0.020	0.050 ± 0.009	0.354 ± 0.088
Spleen	0.045 ± 0.029	0.030 ± 0.006	0.048 ± 0.006	0.233 ± 0.183	0.039 ± 0.003	1.353 ± 0.443
Stomach	0.051 ± 0.027	0.020 ± 0.006	0.110 ± 0.049	0.014 ± 0.006	0.039 ± 0.009	0.032 ± 0.002
Sml. Int.	0.070 ± 0.060	0.016 ± 0.003	0.041 ± 0.008	0.151 ± 0.013	0.027 ± 0.011	0.228 ± 0.058
Lrg. Int.	0.152 ± 0.158	0.033 ± 0.036	1.883 ± 1.670	0.016 ± 0.039	0.035 ± 0.013	0.014 ± 0.008
Kidney	3.076 ± 2.466	0.398 ± 0.012	0.909 ± 0.098	0.281 ± 0.050	0.614 ± 0.078	0.927 ± 0.285
Pancreas	0.082 ± 0.063	0.015 ± 0.006	0.033 ± 0.023	0.011 ± 0.000	0.024 ± 0.006	0.026 ± 0.023
Brain	0.007 ± 0.004	0.003 ± 0.001	0.006 ± 0.003	0.003 ± 0.001	0.004 ± 0.001	0.001 ± 0.007
Bone	0.580 ± 0.162	0.002 ± 0.001	0.053 ± 0.019	0.018 ± 0.005	0.149 ± 0.063	0.079 ± 0.026
Muscle	0.021 ± 0.015	0.007 ± 0.003	0.018 ± 0.008	0.003 ± 0.001	0.014 ± 0.004	0.013 ± 0.006
Fat	0.028 ± 0.013	0.005 ± 0.001	0.032 ± 0.015	—	0.009 ± 0.005	—

TABLE 2
The Biodistribution of the 3 Methylene-Phosphonate Substituted Lanthanide DOTA-Tetraamide Complexes 2 h PostInjection Expressed as % Injected Dose/Organ. Data for ¹⁷⁷Lu4 and ¹⁷⁷Lu6 Were Not Acquired

	¹⁵³ Gd1 ⁺	¹⁷⁷ Lu2 ⁻	¹⁷⁷ Lu3 ³⁺	¹⁷⁷ Lu5 ³⁺
Blood	1.430 ± 0.796	0.060 ± 0.026	0.485 ± 0.091	0.466 ± 0.062
Heart	0.024 ± 0.010	0.005 ± 0.002	0.019 ± 0.017	0.016 ± 0.004
Lung	0.114 ± 0.071	0.020 ± 0.004	0.088 ± 0.034	0.052 ± 0.004
Liver	0.367 ± 0.114	0.248 ± 0.033	0.687 ± 0.150	0.304 ± 0.051
Spleen	0.033 ± 0.023	0.017 ± 0.003	0.027 ± 0.003	0.019 ± 0.003
Stomach	0.089 ± 0.046	0.026 ± 0.010	0.138 ± 0.060	0.045 ± 0.016
Small. Int.	0.092 ± 0.064	0.028 ± 0.010	0.113 ± 0.037	0.117 ± 0.069
Large. Int.	0.270 ± 0.258	0.171 ± 0.110	11.710 ± 10.453	0.261 ± 0.126
Kidney	7.327 ± 5.678	0.264 ± 0.007	0.597 ± 0.046	0.449 ± 0.071
Pancreas	0.066 ± 0.046	0.003 ± 0.001	0.007 ± 0.004	0.017 ± 0.005
Brain	0.007 ± 0.005	0.003 ± 0.001	0.007 ± 0.003	0.005 ± 0.002
Bone	32.927 ± 8.448	0.036 ± 0.027	0.998 ± 0.359	2.747 ± 1.144
Muscle	2.807 ± 1.862	0.072 ± 0.030	0.178 ± 0.191	0.070 ± 0.066
Fat	0.651 ± 0.282	0.121 ± 0.026	0.774 ± 0.362	0.194 ± 0.092

TABLE 3

Pharmacokinetic Parameters From the Fitting of the Data in Figure 3 to a Two-Compartment Model

	¹⁵³ Gd1	¹¹¹ InDTPA	Ratio
$\alpha t_{1/2}$ (min)	3.37 (0.20)	3.99 (0.49)	0.85 [*] (0.06)
$\beta t_{1/2}$ (min)	41.7 (12.0)	56.0 (17.9)	0.75 [*] (0.05)
Elimination $t_{1/2}$ (min)	12.8 (2.7)	20.7 (5.68)	0.62 [*] (0.05)
AUC _{0-∞} (min · kg/L)	196 (49)	240 (76)	0.82 [*] (0.08)
Vol dist (mL/kg)	248 (21)	294 (12)	0.84 [*] (0.04)
MRT (min)	49 (16)	71 (24)	0.70 [*] (0.04)
Clearance (mL/min/kg)	5.4 (1.6)	4.5 (1.7)	1.20 [*] (0.11)

$\alpha t_{1/2}$ = half-life for distribution phase; $\beta t_{1/2}$ = half-life for elimination phase; Vol dist = volume of distribution; AUC_{0-∞} = area under curve from time zero to infinity; MRT = mean residence time in blood.

* indicates $P < 0.002$ by paired Student t test.