Amide conjugates of the DO3A-$N$-(α-amino)propionate ligand: leads for stable, high relaxivity contrast agents for MRI?


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Keywords: DO3A-$N$-(α-amido)propionate chelators; Gd(DO3A-$N$-(α-benzoyleamido)propionate) chelate; fast water exchange; pH stability; safety; in vivo MRI

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Abstract

A new synthetic methodology for preparing amide conjugates of the DO3A-\(N\)-(\(\alpha\)-amino)propionate chelator is disclosed in this manuscript. The synthesis of the DO3A-\(N\)-(\(\alpha\)-benzoylamido)propionate chelator is described as an illustrative example. The model Gd(DO3A-\(N\)-(\(\alpha\)-benzoylamido)propionate) chelate displays accelerated water exchange, stability in a wide pH range and inertness towards transmetallation by Zn\(^{2+}\). The Gd(DO3A-\(N\)-(\(\alpha\)-benzoylamido)propionate) complex is mainly excreted via the kidneys, producing a significant increase of the kidney medulla/cortex enhancement ratio in MR images of Wistar rats, reflecting probably its increased hydrophobicity compared to Gd(DTPA). The results suggest that Gd(DO3A-\(N\)-(\(\alpha\)-amido)propionate) chelates are valuable leads for preparing safe high relaxivity MRI contrast agents.
**Introduction**

Since its introduction 20 years ago, MRI has evolved into the leading imaging modality both in the clinics and in biomedical sciences (1). Constant developments in instrumentation, particularly the use of increasingly higher magnetic fields, and the use of contrast agents (CAs), have driven MRI to the forefront of medical imaging (2). Gd$^{3+}$ chelates are the most useful CAs for MRI due to the high paramagnetism (7 unpaired electrons) and long electronic relaxation times of the Gd$^{3+}$ ion (3). To ensure safety, Gd$^{3+}$ ions are used as complexes with poly(aminocarboxylate) ligands, which prevents the inherent toxicity of the free metal ion and modulates its unfavorable pharmacokinetic behaviour. The efficacy of a CA is measured by its relaxivity ($r_1$, $r_2$)-the enhancement of the relaxation rate of the water protons brought about by a 1 mM concentration of Gd$^{3+}$ (4,5). Increasing detection sensitivity, by maximizing relaxivity, has been the driving force in the development of new CAs (6). Nonetheless, the high relaxivities predicted theoretically haven’t yet been achieved in spite of the tremendous efforts and developments in the field (7). The main approaches to enhance detection sensitivity involve optimizing the relaxivity of individual chelates, eg. through clustering a high number of (high relaxivity) chelates into (bio)macromolecular carriers or self-assembly of monomeric chelates into supramolecular structures, and directing chelate constructs to the sites of interest by attaching targeting moieties recognized specifically by cellular receptors (6,8). All these approaches require chemical modification of the basic chelator structure in order to introduce “handles” for chemical functionalization.

A few years ago, the discovery of severe toxicity associated with repeated CA MRI in individuals suffering from kidney function impairment shaded doubts about the safety of MRI CAs (9). This condition, named Nefrogenic Systemic Fibrosis (NSF), due to generalized formation of fibrotic (scarring) plates in the skin and internal organs, was correlated with the use of Gd$^{3+}$ chelates of DTPA bis-amide chelators (10). Release of Gd$^{3+}$ ions *in vivo*, due to insufficient kinetic and thermodynamic stability of the Gd(DTPA-bis-amide) chelates, seems the most likely reason for CA toxicity (11,12). Importantly, no cases have been associated with CAs of the DOTA family, even in patients suffering from kidney disease (12). The much higher kinetic and thermodynamic stability of the macrocyclic DOTA-type CAs precludes the release of Gd$^{3+}$ ions *in vivo*. The adoption of stricter guidelines concerning the use of Gd$^{3+}$-based CA has drastically reduced the number of NSF cases reported (13). Nonetheless, safety,
associated with chelate stability, rather than high relaxivity (high detection sensitivity), is becoming the new paradigm in CA MRI, which favours the use of macrocyclic, DOTA-type CAs. There are several CAs of this type approved for clinical use in Europe, most of which are extracellular CAs. Gd(HPDO3A) (Prohance®) has become the CA of choice in the clinical setting (14). Improving the performance of DOTA-type CAs (targeting, multimerization, responsiveness, etc.) requires chemical elaboration of the basic DOTA scaffold to allow conjugation to (bio)molecules. Endowing DOTA chelators with conjugability without compromising stability is a core problem in CA research. In fact, the most convenient route for chemical functionalization of the DOTA skeleton, differentiating an acetate arm for amide coupling, reduces the thermodynamic and kinetic inertness of Gd(DOTA-monoamide)-type chelates (15). Furthermore, neutral Gd(DOTA-monoamide)-type chelates display much slower water exchange, limiting the relaxivities potentially achievable by chelates displaying optimized rotational correlation times ($\tau_R$). Introducing pendant functional groups on the ethylenediamine bridges on the DOTA skeleton is an effective, although costly solution (16). It is preferable from the synthetic point of view to elaborate the (pre-formed) available cyclen skeleton than starting chelator synthesis from scratch. We have recently reported the synthesis of a new chelator, DO3A-$N-(\alpha$-amino)propionate (L1, Scheme 1) and the relaxometric characterization of its Gd$^{3+}$ complex. The $\alpha$-amino substituted propionate arm presumably imposes steric compression around the water binding site on the complex, resulting in an accelerated water exchange rate $k_{ex}$ (17). The $k_{ex}$ value for the Gd(DO3A-$N-(\alpha$-amino)propionate) (GdL1) chelate is of the same order of magnitude as that reported for its (unsubstituted) propionate analogue Gd(DOTA-$N$-propionate)- in the range considered ideal for attaining high relativity at intermediate/high field (15,18). Moreover, the GdL1 complex is stable in the pH range 2-10 and inert towards transmetallation with Zn$^{2+}$. The pendant available amine group is ideal for conjugation through a variety of ligation chemistries. However, an important issue remains to be addressed: what is the effect of amide conjugation on the stability and water exchange rate of Gd(DO3A-$N-(\alpha$-amido)propionate) chelates? In this manuscript we describe an expeditious methodology for the synthesis of amide conjugates of the DO3A-$N-(\alpha$-amino)propionate chelator. The relaxometric characterization and in vivo MRI evaluation of a model amide chelate, Gd(DO3A-$N-(\alpha$-benzoylamido)propionate) (GdL1), is reported.
Results and Discussion

Synthesis

Allying stability to conjugability is an important requisite for developing metal chelates into imaging and/or therapeutic agents. We have proposed before that the pendant amine group on the α-aminopropionate arm on the DO3A-N-α-aminopropionate chelator is an ideal handle for conjugation to (bio)molecules (17). Fully deprotected LnL₂ chelates (Scheme 1) could be used directly for conjugation (19). However, solubility issues and difficult manipulations and purifications can be avoided, especially with radio-labelled metal chelates, by labelling pre-formed fully deprotected conjugates. Scheme 1 discloses a new expeditious strategy for the synthesis of amide conjugates of the DO3A-N-(α-amino)propionate scaffold.

Scheme 1. Synthetic sequence for preparing the DO3A-N-(α-benzoylamido)propionate ligand and its Gd³⁺ chelate. a) K₂CO₃, MeCN; b) i. TFA/DCM, ii. K₂CO₃/ethylbromoacetate, MeCN; c) i. Dowex 1X2100-OH- resin, ii. Elution with hydrochloric acid 0.1 M; d) GdCl₃.xH₂O.

Introducing a pre-formed Boc-dehydroamide (Ph, Boc)-Δ-AlaOMe 2 into the cyclen scaffold via Michael addition is the key step in this reaction sequence. Building up on our previous experience on the synthesis of bifunctional chelator 8 we envisaged that (Boc, amide)-dehydroalanines of type 2 could fulfil electronic and steric
requirements to perform well as electrophiles in Michael addition to cyclen. (Boc, amide)-dehydrolalanines of type 2 can be readily prepared in two steps from serine methyl ester hydrochloride 1 and stored at room temperature for extended periods (20). Michael addition of block (2) to cyclen 3 proceeded smoothly in acetonitrile, using K₂CO₃ as base, in 2-3 hours. An excess of cyclen, 1.5 eq., was used in order to bias the statistical mixture towards the monoalkylated product 4. Removing the Boc group at the monoalkylated intermediate 4 stage could avert retro-elimination during the next alkylation step. In fact, one pot treatment of 4 with TFA, followed by alkylation with ethyl bromoacetate in the presence of a large excess of K₂CO₃ produced the fully alkylated amide 5 in moderate yield without evidence of retro-elimination. One step deprotection of the fully alkylated pro-chelator 5 with the ion-exchange resin Dowex 1×2 OH⁻ followed by elution with hydrochloric acid yielded the fully deprotected DO3A-N-(α-benzoylamido)propionate chelator 6 in overall non-optimised yield of 16% over 3 steps. Importantly, this approach is general, applicable to Boc-dehydroamides of type 2 lacking acid sensitive moieties or acid sensitive protecting groups on the amide moiety.

**Solution NMR and relaxometric studies**

As discussed above, the aim of this work is to ascertain if Gd³⁺ chelates of amide conjugates of the DO3A-N-(α-amino)propionate metal chelator retain the high stability, inertness and fast water exchange of its parent GdL₂ complex. The soluble, low molecular weight Gd³⁺ chelate 7 (Scheme 1) was selected as a model system in order to avoid solubility and self-assembly complicating issues.

¹H NMR spectra (600 MHz) of LnL₁ complexes (Ln³⁺ = La, Sm and Eu) at 298 K and neutral pH were found to contain very broad resonances for the macrocyclic and pendant arm methylene protons (data not shown), indicating the presence of an internal isomerisation process at intermediate exchange on the NMR time scale (21). The spectra of the parent LnL₂ complexes are sharp in the same conditions (17).

The water exchange rate, kₑₓ⁹⁸, for the GdL₁ complex was determined from variable-temperature ¹⁷O NMR studies (Figure 1 a-b). Additionally, the magnetic field dependence of the longitudinal water proton relaxivities (r₁) was measured by ¹H nuclear magnetic relaxation dispersion (NMRD) at 298 and 310 K (Figure 1c) with the objective of determining the parameters that describe water exchange and rotational dynamics for the GdL₁ complex.
Figure 1. Temperature dependence of the (a) reduced transverse and longitudinal \(^{17}\)O relaxation rates \(1/T_{2r}\) and \(1/T_{1r}\), and (b) chemical shifts \(\Delta \omega_r\): \(B = 9.4\ T (\ln(1/T_{1r}), (■)), (\ln(1/T_{2r}), (▲)),\) and (c) NMRD profiles (298 K (▲) and 310 K (■)) for the Gd(DO3A-N-(α-benzoylamido)propionate) complex.

The Solomon-Blombergen-Morgan theory was used for analyzing simultaneously the experimental \(^1\)H NMRD data, as well as the \(^{17}\)O NMR reduced longitudinal (\(1/T_{1r}\)) and transverse (\(1/T_{2r}\)) relaxation rates and chemical shifts (\(\Delta \omega_r\)) (5). In the analysis, the following parameters have been fitted: rate (\(k_{ex}^{298}\)), activation enthalpy (\(\Delta H^\ddagger\)) and
activation entropy ($\Delta S^\ddagger$) of the water exchange, the rotational correlation time ($\tau_{RH}^{298}$) and its activation energy ($E_R$), the electron spin relaxation parameters ($\tau_v^{298}$, $\Delta^2$ and $E_V$) and the scalar coupling constant ($A/\hbar$). The diffusion constant and its activation energy have been fixed to $D_{GdH298} = 26 \times 10^{-10} \text{ m}^2\text{s}^{-1}$ and $E_{DGdH}^{298} = 22 \text{ kJ/mol}$, respectively. The Gd-water proton distance was fixed to $r_{GdH} = 3.1$ Å, and the distance of closest approach between the Gd$^{3+}$ ion and the outer sphere protons to $a_{GdH} = 3.6$ Å (17,18,22). The parameters obtained from the simultaneous fitting of the data are shown on Table 1, and the corresponding fitted curves are shown in Figure 1.

### Table 1. Best fit parameters obtained for the Gd(DO3A-N-(α-benzoylamido)propionate) chelate from the simultaneous fitting of the NMRD data, $^{17}$O NMR longitudinal ($1/T_1r$) and transverse ($1/T_2r$) relaxation rates and chemical shifts ($\Delta\omega_r$).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>GdL$_1^a$</th>
<th>GdL$_2^b$</th>
<th>Gd(DOTA)$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$g(H_2O)$</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>$\Delta H^\ddagger_r$ [$\text{J/K/mol}$]</td>
<td>17±1.1</td>
<td>19.1</td>
<td>54.5</td>
</tr>
<tr>
<td>$\Delta S^\ddagger_r$ [$\text{kJ/mol}$]</td>
<td>-40±6</td>
<td>-35</td>
<td>+65</td>
</tr>
<tr>
<td>$k_{ex}^{298}$ [$10^{-7} \text{ s}^{-1}$]</td>
<td>5.14 ± 0.4</td>
<td>4</td>
<td>0.46</td>
</tr>
<tr>
<td>$E_R$ [$\text{kJ/mol}$]</td>
<td>26.2±1.1</td>
<td>17</td>
<td>20</td>
</tr>
<tr>
<td>$\tau_{RH}^{298}$ [$\text{ps}$]</td>
<td>182±10</td>
<td>71</td>
<td>100</td>
</tr>
<tr>
<td>$E_V$ [$\text{kJ/mol}$]</td>
<td>4.8±0.5</td>
<td>1</td>
<td>8.6</td>
</tr>
<tr>
<td>$\tau_v^{298}$ [$\text{ps}$]</td>
<td>7.2±1.5</td>
<td>11</td>
<td>0.65</td>
</tr>
<tr>
<td>$\Delta^2$ [$10^{-20} \text{ s}^{-2}$]</td>
<td>0.5±0.06</td>
<td>0.25</td>
<td>-</td>
</tr>
<tr>
<td>$A/\hbar$ [$10^6 \text{ rad/s}$]</td>
<td>-3.13±0.4</td>
<td>-3.6</td>
<td>-4.0</td>
</tr>
</tbody>
</table>

*Parameters underlined have been fixed. From ref. (17). From ref. (22).* 

In the fitting, one inner sphere water molecule was assumed in analogy with the parent GdL$_2$ complex (17). This assumption was confirmed by the value obtained for the parameter $A/\hbar$, characteristic of $q = 1$ complexes of the DOTA family. The rate constant for water exchange for the GdL$_1$ complex ($k_{ex}^{298} = (5.14 \pm 0.4) \times 10^7 \text{ s}^{-1}$) is of the same order of magnitude as that reported for its parent GdL$_2$ complex ($k_{ex}^{298} = (4.0 \pm 0.4) \times 10^7 \text{ s}^{-1}$) (17) and for the unsubstituted Gd(DOTA-N-propionate) complex ($k_{ex}^{298} = 6.1 \times 10^7 \text{ s}^{-1}$) (15). Moreover, $k_{ex}$ is around one order of magnitude higher than that reported for Gd(DOTA) (22). This result confirms our working hypothesis, that Gd$^{3+}$ chelates of amide conjugates of the DO3A-N-(α-amino)propionate chelator retain the fast water exchange displayed by its parent GdL$_2$ complex. This is an important
result, indicating that amide conjugates displaying an optimized rotational correlation time, $\tau_R$, could attain high relaxivities at intermediate fields, as the water exchange rate is in the ideal range. The value for the rotational correlation time obtained from the $^1$H longitudinal relaxation rates ($\tau_{R^{298H}} = 182$ ps) is characteristic of low molecular weight chelates, indicating that the complex does not self-assemble in the concentration range studied. At high magnetic fields, the relaxivity is dominated by fast rotation in solution, as demonstrated by the temperature dependence of the relaxivity.

Complex stability is of paramount importance for meaningful biological applications. The NSF condition results, probably, from complex dissociation in the challenging biological medium. It is widely accepted that transmetallation with endogenous metal ions, the most abundant of which in blood serum is Zn$^{2+}$, and ligand protonation assisted demetallation, at low pH, are the most likely mechanisms for Gd$^{3+}$ leakage(23). The kinetic inertness of the GdL$_1$ complex was evaluated using the transmetallation assay developed by Muller and co-workers (24), in which complex transmetallation by Zn$^{2+}$ was studied in phosphate-buffer (10 mM, pH 7.1) (Figure 2).

![Figure 2](image_url)

**Figure 2.** Evolution of the relative water proton paramagnetic relaxation rate $R_{1p}(t)/R_{1p}(0)$ (20 MHz, pH 7.1, 298 K) versus time for a 1.5 mM solution of Gd(DO3A-$N$-(α-benzyolamido)propionate complex in phosphate buffer (10 mM, pH 7.1) (○) and in phosphate buffer containing an equimolar amount of Zn$^{2+}$ ions (●).

Replacement of Zn$^{2+}$ by Gd$^{3+}$ in the initial complex would generate a time-dependent decrease of the paramagnetic contribution to the water proton spin-lattice relaxation rate ($R_{1p}$) due to precipitation of GdPO$_4$. The time evolution of the relative water proton paramagnetic relaxation rate $R_{1p}(t)/R_{1p}(0)$ versus time for the GdL$_1$
complex in phosphate buffer, in the absence and in the presence of Zn\(^{2+}\) (Figure 2) surpasses the accepted empirical criteria for kinetic stability and thermodynamic stability (24). Furthermore, the independence of the paramagnetic relaxation rates for the GdL\(_1\) complex from the solution pH (Figure 3) confirms that the complex is stable in the wide pH range 2-11.

![Graph](image)

**Figure 3.** pH dependence of the relative water proton paramagnetic relaxation rate \(R_{1p}\) (20 MHz, 25 °C) for a 1.5 mM solution of Gd(DO3A-N-(\(\alpha\)-benzoylamido)propionate.

The interaction of the GdL\(_1\) complex with human serum albumin (HSA) was studied by relaxometric titration (Fig. S1, Supplementary Information). A very weak binding was detected, which is not likely to affect the pharmacokinetic behaviour of the complex *in vivo*.

**MRI Studies**

The GdL\(_1\) contrast agent was well tolerated by the rats. Their body temperature remained stable and no alteration on the body functions was observed during the course of the experiment.

Series of T\(_1\)-weighted spin-echo axial images of the DCE MRI experiments were obtained with the GdL\(_1\) complex (dose 0.1 mmol/kg body weight) and, for comparison purposes, with the Gd(DTPA) complex (dose 0.1 mmol/kg body weight). As can be seen (Figure 4A and B), both complexes follow mainly renal elimination with a very small hepatobiliary contribution to excretion.
The features of the data in Figure 4 are better understood as illustrated in Figure 5, which shows their quantitative analysis. Each time point corresponds to the average intensity within ROIs placed on the different organs. In order to compare the results for all the animals under study (n = 4), the data were normalized by calculating the mean relative enhancement of each ROI. Muscle and liver enhancement is comparably low with both complexes. The relative enhancement obtained with GdL is increased almost immediately after intravenous injection from 0 up to about 250% in the kidney medulla and 200% in the kidney cortex, followed by a first order-like decrease to values around 120% and 80%, respectively, after 50 minutes. The time course of the average relative enhancement after injection of Gd(DTPA) (Figure 5B) is in good agreement with the literature (25). The renal pharmacokinetic behavior of Gd(DTPA) is significantly different from that observed for the GdL complex. Kidney cortex enhancement is much less abrupt than for GdL, reaching a maximum of about 100% after 10 minutes post injection, followed by a steady decrease to a value (~80%) comparable to that reached by GdL after 50 minutes.
Figure 5. Time course of signal intensity, relative to the initial value (up to 50 min post-injection) of several regions of interest during dynamic contrast enhancement MRI experiments in rats with (A) GdL₁ (0.1 mmol kg⁻¹ BW) and (B) Gd(DTPA) (0.1 mmol kg⁻¹ BW). The time courses are data for mean values of four animals and the corresponding standard error.

However, the main difference between Gd(DTPA) and GdL₁ regards kidney medulla enhancement. While for the GdL₁ complex there is a sharp increase just after injection, followed by a first order-like decrease, for Gd(DTPA) a saturation-like kinetic behaviour is observable. After 50 minutes, a significantly higher enhancement of the kidney medulla is attained with the Gd(DTPA) comparing to GdL₁ complex (200% vs 120%). As a consequence of the disparate pharmacokinetics, the ratio of medulla/cortex enhancement is approximately constant for the GdL₁ complex over the duration of the experiment, while it increases significantly for the Gd(DTPA) complex. As both complexes share the same overall (-1) charge and plausibly a similar chelate geometry, the observed difference of pharmacokinetic behaviour is likely to be due to the increased hydrophobicity of the GdL₁ complex.

Conclusions
In this manuscript we demonstrate that Gd³⁺ complexes of amide conjugates of the DO3A-N-(α-amido)propionate chelator retain the fast water exchange and stability of their parent DO3A-N-(α-amino)propionate chelator. An expeditious, convergent synthetic methodology for DO3A-N-(α-amido)propionate chelators is illustrated by the synthesis of the model chelator DO3A-N-(α-benzoylamido)propionate. DCE-MRI studies in rats revealed that the Gd(DO3A-N-(α-benzoylamido)propionate) chelate is mainly excreted via the kidneys. A significant increase of the enhancement ratio kidney medulla/kidney cortex is produced by the contrast agent after 50 minutes, reflecting probably its increased hydrophobicity comparing to Gd(DTPA).
The results presented here suggest that Gd(DO3A-N-(α-amido)propionate) chelates are valuable leads for preparing safe high relaxivity MRI CAs. In fact, Gd(DO3A-N-(α-amido)propionate) chelates with optimized water exchange features, when bound to suitable carriers, are likely to originate high relaxivities at intermediate fields by virtue of the simultaneous optimisation of $k_{ex}$ and $\tau_R$. The high relaxivities of such imaging platforms allied to the chelate stability and safety could find many uses in biomedical and pre-clinical diagnostic MRI applications.

**Experimental**

**Materials and Methods**

Chemicals were purchased from Sigma-Aldrich and used without further purification. Solvents used were of reagent grade and purified by usual methods. Cyclen was purchase from CheMatech. Reactions were monitored by TLC on Kieselgel 60 F254 (Merck) on aluminium support. Detection was by examination under UV light (254 nm) and by adsorption of iodine vapour. Flash chromatography was performed on Kieselgel 60 (Merck, mesh 230–400). The relevant fractions from flash chromatography were pooled and concentrated under reduced pressure, $T < 313$ K. Ion exchange chromatography was performed on Dowex1X2100-OH− resin (Sigma Aldrich). The resin was purchase as the Cl− form and converted to the OH− form by the standard procedure. $^1$H and $^{13}$C NMR spectra (assigned by 2D DQF-COSY and HMQC techniques) were run on a Varian Unity Plus 300 NMR spectrometer, operating at 299.938 MHz and 75.428 MHz, for $^1$H and $^{13}$C, respectively. Chemical shifts ($\delta$) are given in ppm relative to the CDCl$_3$ solvent ($^1$H, $\delta$ 7.27; $^{13}$C, $\delta$ 77.36) as internal standard. For $^1$H and $^{13}$C spectra recorded in D$_2$O, chemical shifts ($\delta$) are given in ppm relative to TSP as internal reference (1H, $\delta$ 0.0) and tert-butanol as external reference ($^{13}$C, CH$_3$ $\delta$ 30.29).

**Synthesis of monoalkylated cyclen (4):** K$_2$CO$_3$ (4.07 g, 29.46 mmol) was added to a solution of cyclen (1.27 g, 7.37 mmol) in MeCN (50 cm$^3$). To this solution was added (Ph, Boc)-Δ-AlaOMe 2 (1.5 g, 4.91 mmol). The suspension was stirred vigorously at room temperature for 3 hrs. The suspension filtered and the filtrate was evaporated under reduced pressure. The residue was purified by flash chromatography (100% CH$_2$Cl$_2$ $\rightarrow$ CH$_2$Cl$_2$/EtOH/NH$_3$/H$_2$O (50:50:1:1)) to afford compound 4 as a viscous light
yellow oil (1.34 g, 59.1%). $^1$H NMR (300 MHz, CDCl$_3$): $\delta = 1.47$ (s, 9H, Boc), 2.50-2.90 (m, 16H, N(CH$_2$)$_2$N), 3.01 (dd, $J = 14.1$ and 6 Hz, 1H, NCH$_2$H$_2$CH), 3.49 (dd, $J = 14.4$ and 5.1 Hz, 1H, NCH$_2$H$_2$CH), 3.76 (s, 3H, OMe), 5.25 (t, $J = 6$ and 5.1 Hz, 1H, NCH$_2$H$_2$CH), 7.30-7.70 (5H, m, Ar). $^{13}$C NMR (75.4 MHz, CDCl$_3$): $\delta = 27.27$ (C(CH$_3$)$_3$), 44.77 (CH$_2$), 45.83 (CH$_2$), 46.70 (CH$_2$), 51.27 (CH$_2$), 52.51 (OCH$_3$), 55.61 (NCH$_2$CH), 56.08 (CH), 83.88 (C(CH$_3$)$_3$), 127.87 (Ar), 127.96 (Ar), 131.27 (Ar), 137.02 (Ar), 152.72 (NC(O)O), 170.57 (C(O)Ar), 172.62 (C(O)OCH$_3$). HRMS (ESI): m/z: cacad for C$_{24}$H$_{39}$N$_5$O$_5$: 478.3029, found: 478.3024.

**Synthesis of tetraalkylated cyclen (5):** A solution of monoalkylated cyclen 4 (1.30 g, 2.72 mmol) in trifluoroacetic acid in dichloromethane (60%, 25 cm$^3$) was stirred overnight at room temperature. The solvent was evaporation at reduced pressure and the residue was redissolved in dichloromethane. The solvent was evaporated, and this procedure was repeated several times to give a light yellow thick oil. $^1$H NMR spectroscopy (CDCl$_3$) revealed the disappearance of the signals assigned to the Boc groups in the precursor compound 4. The deprotection was assumed as quantitative. To a solution of Boc-deprotected compound 4 (2.72 mmol, assuming quantitative deprotection) in MeCN (40 cm$^3$) was added K$_2$CO$_3$ (4.51 g, 32.6 mmol). The suspension was vigorously stirred for 30 minutes at room temperature before adding ethyl bromoacetate (1.06 cm$^3$, 9.52 mmol). The suspension was further stirred at room temperature for 2.5 hrs. The suspended solid was removed by filtration, the filtrate was evaporated under reduce pressure and the residue was purified by flash chromatography (100% CH$_2$Cl$_2$ $\rightarrow$ CH$_2$Cl$_2$/EtOH (1:1)) to afford compound 5 (0.632 g, 37%) as a white foam. $^1$H NMR (300 MHz, CDCl$_3$): $\delta = 1.22$ (m, 9H, CH$_2$CH$_3$), 2.2-3.60 (broad, overlapped signals with a integration corresponding to, 24 H, NCH$_2$ and NCH$_2$H$_2$CH), 3.71 (s, 3H, C(O)OCH$_3$), 5.14 (m (br), 1H, NCH$_2$H$_2$CH), 7.44-8.03 (5H, m, Ar). $^{13}$C NMR (75.4 MHz, CDCl$_3$): selected signals: 13.95 (CH$_2$CH$_3$), 48.74 (CH), 49.04 (CH$_2$), 50.38 (CH$_2$), 51.48 (CH$_2$), 52.59 (CH$_2$), 52.95 (OCH$_3$), 53.62 (CH$_2$CH), 53.62 (CH$_2$), 54.86 (CH$_2$), 54.96 (CH$_2$), 55.19 (CH$_2$), 55.37 (CH$_2$), 55.75 (CH$_2$), 56.18 (CH$_2$), 60.62 (CH$_2$), 60.94, 61.13, 61.21 (OCH$_2$), 127.83 (Ar), 128,12 (Ar), 128,41 (Ar), 131,53 (Ar), 131,84 (Ar), 132,62 (Ar), 167.79 (C(O)Ar), 170.29 (2X C(O)OCH$_3$), 170.52 (C(O)OCH$_3$), 170.91 (C(O)OCH$_2$). HRMS (ESI): m/z: cacad for C$_{31}$H$_{49}$N$_5$O$_5$: 636.3609, found: 636.3590.
Fully deprotected DO3A-N-(α-benzoylamido)aminopropionate metal chelator (6):

Compound 5 (0.566 g, 0.89 mmol) was dissolved in a mixture made up of 20 cm$^3$ of water and 20 cm$^3$ of ethanol. The solution was adjusted to pH ~ 10-11 by adding small portions of Dowex 1X2-100-OH$^-$ resin. The suspension was kept under stirring at room temperature for 2 hours. The wet resin was transferred into a chromatography column, washed with water (~50 cm$^3$) and eluted with 0.1 M hydrochloric acid. The relevant fractions, identified by TLC (ethanol water 1/1, revelation with iodine vapor) were pooled, concentrated at room temperature and further dried under vacuum to afford the final deprotected compound, in the hydrochloride form, as a light yellow solid (6) (0.276 g, 71.2%).

$^1$H NMR (300 MHz, CDCl$_3$): $\delta$ = 2.9-3.6 (broad, overlapped signals with integration corresponding to, 18 H, N(CH$_2$)$_2$N and NCH$_2$), 3.81 (broad, overlapped signals with integration corresponding to, 6 H, NCH$_2$), 4.80 (m (br), $^1$H, CH), 7.53-7.88 (5H, m, Ar). $^{13}$C NMR (75.4 MHz, CDCl$_3$): selected signals: 43.02 (CH$_2$), 48.11 (CH$_2$), 48.93 (CH$_2$), 49.03 (CH$_2$), 49.78 (CH$_2$), 51.39 (CH$_2$), 51.71 (CH), 51.91 (CH$_2$), 54.53 (CH$_2$), 56.08 (CH$_2$), 56.77 (2x CH$_2$), 127.46 (2x Ar), 129.04 (2x Ar), 132.60 (Ar), 133.32 (Ar), 170.20 (C(O)), 177.17 (C(O)Ar). HRMS (ESI): m/z: cacd for C$_{24}$H$_{36}$N$_5$O$_9$: 538.2513, found: 538.2508.

$^1$H and $^{17}$O NMR and $^1$H NMRD experiments

Sample preparation

To an aqueous solution of the ligand (pH = 5.0) was added drop-wise an aqueous solution of the corresponding LnCl$_3$.xH$_2$O in a 1:1 mole ratio. The solution was stirred at room temperature over 1 hour while keeping its pH at around 5.7 by adding aqueous KOH. The solution was concentrated under reduced pressure to afford off white solids. The solutions for NMR measurements were obtained by dissolution of the appropriate amounts of solid complexes in D$_2$O ($V = 1$ cm$^3$) to obtain 20 mM concentrations. Proton 1D spectra of the solutions of the paramagnetic (Sm$^{3+}$ and Eu$^{3+}$) and diamagnetic (La$^{3+}$) complexes were obtained at 298 K on a Varian VNMRS 600 (14.09 T, 600.14 MHz) NMR spectrometer.

The solutions of the GdL$_1$ complex for $^{17}$O NMR and $^1$H NMRD experiments were prepared by mixing equimolar amounts of GdCl$_3$ and ligand. A slight excess (5%) of ligand was used. The solution was adjusted to pH ~5.8 with aqueous NaOH and was allowed to react for 24 hours at room temperature. The absence of free metal was
checked in each sample by testing with xylenol orange (22,26,27). The pH of the stock solution was adjusted by adding aqueous NaOH (0.1 mM). $^{17}$O-enriched water ($^{17}$O: 11.4%) was added to the solutions for $^{17}$O measurements to improve sensitivity. The final concentration of the complex solution was 17.56 mmol kg$^{-1}$ at pH = 6.90. For the NMRD experiments a 5.00 mM solution of complex at pH 6.98 was used.

$^{17}$O NMR experiments

Variable-temperature $^{17}$O NMR measurements were performed on a Bruker Avance-500 (11.7 T) spectrometer. A BVT-3000 temperature control unit was used to stabilize the temperature, measured by a substitution technique. The samples were sealed in glass spheres that fitted into 10 mm o.d. NMR tubes, to eliminate susceptibility corrections to the chemical shifts (28). Longitudinal relaxation rates ($1/T_1$) were obtained by the inversion recovery method, and transverse relaxation rates ($1/T_2$) by the Carr-Purcell-Meiboom-Gill spin-echo technique. As an external reference, acidified water of pH 3.4 was used.

NMRD measurements

The measurements were performed by using a Stelar Spinmaster FFC NMR relaxometer (0.01–20 MHz) equipped with a VTC90 temperature control unit. At higher fields, the $^1$H relaxivity measurements were performed on a Bruker Electromagnet at the frequencies of 30 MHz, 40 MHz, 60 MHz and 80 MHz. In each case, the temperature was measured by a substitution technique. Variable temperature measurements were performed at 25 and 37°C.

Relaxivity studies of pH dependence and Zn$^{2+}$ transmetallation

The transmetallation reaction of GdL$_1$ with Zn$^{2+}$ was studied by the time dependent decrease of the water proton longitudinal relaxation rate, $R_1$, measured on a Bruker Minispec mq20 (20 MHz, 298 K) relaxometer, of a phosphate-buffered saline solution (PBS, pH 7.1, 10 mM) containing 1.5 mM of GdL$_1$ after adding an equimolar amount of ZnCl$_2$, while the sample was vigorously stirred (29). The water longitudinal relaxation rate was also measured as a function of time on the PBS buffered solution (pH 7.1, 10 mM) containing 2.5 mM GdL$_1$ (30). The pH dependence of the relaxivity of the GdL$_1$ solution was also measured on the same relaxometer.
MRI experiments

In vivo MRI studies. The experimental protocols performed were approved by the appropriate institutional review committees and meet the guidelines of their responsible governmental agency. The Magnetic Resonance Imaging (MRI) experiments were all performed on a Bruker Pharmascan platform (Bruker Medical Gmbh, Ettlingen, Germany) using a 7.0-T horizontal-bore superconducting magnet, equipped with a $^1$H selective 60 mm birdcage resonator and a Bruker gradient insert with 90 mm diameter (maximum intensity 360 mT/m). Data were acquired using a Hewlett-Packard console running Paravision software (Bruker Medical Gmbh, Ettlingen, Germany) under a LINUX environment.

All MRI examinations were carried out on male Wistar rats ($n = 4$, 250-260 g body weight) anaesthetized initially by inhalation in an induction box with O$_2$ (1 l/min) containing 3 % isoflurane, and maintained during the experiment using a mask and 1-2 % isoflurane on O$_2$. Animals were taped down into a holder, to minimize breathing-related motion, and were then placed in a heated probe, which maintained the core body temperature at approx. 37 °C, monitored by a rectal probe. The physiological state of the animal was monitored throughout the entire experiment by a Biotrig physiological monitor (Bruker Medical Gmbh, Ettlingen, Germany), using the respiratory rate and body temperature. 100 mM solutions of Gd(DO3A-N-(α-benzoylamido)propionate and Gd(DTPA) (Magnevist®, Schering, Berlin, Germany) were prepared in distilled water and the pH was adjusted to 7.2. The solutions were injected into the catheterized tail vein as a bolus in 20 s (0.1 mmol Gd.kg$^{-1}$ body weight) using an infusion pump (Panlab, Barcelona, Spain). Dynamic contrast-enhanced (DCE) MRI experiments were performed with series of T$_1$-weighted spin echo images sequentially acquired over 1 h, following the injection of the contrast agent 10 min after the beginning of the study. The acquisition parameters were: TR = 310 ms, TE = 10.58 ms, number of averages = 2, ten coronal slices, slice thickness = 2 mm, FOV = 5.0 x 5.0 cm, matrix = 256 x 256, 30 repetitions with a total acquisition time of 119 s.

MRI Data analysis. Data were analyzed with Image J (http://rsbweb.nih.gov/ij/). With the aim of comparing the pharmacokinetics obtained from different animals, the data were normalized by calculating the percentage of relative, rather than absolute, enhancement:
\[ RE = \frac{(I - I_0)}{I_0} \times 100 \]

where \( I \) is the signal intensity at any given time after CA injection and \( I_0 \) is the intensity before injection. Pharmacokinetic behaviour was analyzed by calculating the average enhancements within different ROIs placed on each one of the following regions: liver, kidney medulla, kidney cortex and muscle.

**Acknowledgements**

This work was financially supported by Fundação para a Ciência e a Tecnologia, Portugal: project PTDC/QUI/70063/2006, including a grant to C.I.O.M., grant SFRH/BD/63994/2009 to M.F.F. and grant SFRH/BD/46370/2008 to A.F.M. and Rede Nacional de RMN (REDE/1517/RMN/2005) for the acquisition of the Varian VNMRS 600 NMR spectrometer in Coimbra. T.B.R. was supported by a Marie Curie Fellowship (FP/-PEOPLE-2009-IEF 254380) and an EMBO Fellowship (ALTF 1145-2009). Financial support from La Ligue Contre le Cancer, France (E.T.), and from Ministerio de Ciencia e Innovación, Spain: projects SAF2011-23622 (S.C.) and CTQ2010-20960-C02-02 (P.L.-L.), and Comunidad de Madrid, Spain, project S2010/BMD-2349 (S.C. and P.L.-L). This work was carried out in the frame of the COST D38 Action “Metal Based Systems for Molecular Imaging” and COST TD1004.

**Supplementary Information.** Figure S1: \( R_{1p} \) dependence on \([GdL1]\) in water in the absence and presence of 4% (0.6 mM) HSA (circles) (20 MHz, 298 K, pH = 7.1). Equations used for the analysis of NMRD and \(^{17}\)O NMR data.
References

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