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Magnetic resonance and optical imaging probes for NMDA receptors on the cell surface of neurons: synthesis and evaluation in cellulo

Neil Sim\textsuperscript{a}, Robert Pal\textsuperscript{a}, David Parker, *\textsuperscript{a} Joern Engelmann \textsuperscript{b}, Anurag Mishra* \textsuperscript{a,c} and Sven Gottschalk\textsuperscript{b,c}

\textsuperscript{a} Department of Chemistry, Durham University, South Road, Durham DH1 3LE, UK
\textsuperscript{b} High Field MR Centre, Max Planck Institute for Biological Cybernetics, Spemannstrasse 41, Tuebingen, D-72076, Germany.
\textsuperscript{c} Institute for Biological and Medical Imaging, Helmholtz Center, Munich, Neuherberg, D-85764, Germany.

email: david.parker@dur.ac.uk; anurag.mishra@helmholtz-muenchen.de

A second generation of N-methyl-D-aspartate (NMDA) receptor-targeted MRI contrast agents has been synthesised and evaluated in cellulo, based on established bicyclic NMDA receptor antagonists. Their use as responsive MR imaging probes has been evaluated in suspensions of NSC-34 cells, and one agent exhibited significant enhancements in measured longitudinal and transverse water proton relaxation rates (19 and 38\% respectively; 3T, 298K). A biotin derivative of the lead compound was prepared and the specificity and reversibility of binding to the NMDA cell surface receptors demonstrated using confocal laser scanning microscopy. Competitive and reversible binding of glutamate to the receptors was also visualised, suggesting that the receptor-targeted approach may allow MRI to be used to monitor neuronal events associated with modulation of local glutamate concentrations.

Introduction

Receptors for N-methyl-D-aspartate (NMDA) are found on the surface of neurons (e.g. astrocytes) and constitute part of the ligand-gated ionotropic family of glutamate receptor proteins.\textsuperscript{1} These receptors play a key role in excitatory neurotransmission and in memory and learning. Many central nervous system (CNS) disorders have been related to the misregulation or over-stimulation of NMDA receptors including epilepsy, ischemia, Parkinson’s and Alzheimer’s diseases.\textsuperscript{2-4} Selective antagonists of the NMDA receptors (NMDAR) have also been studied, exploring their potential as imaging or therapeutic agents for these and related CNS disorders.\textsuperscript{5,6}
One of the goals of molecular imaging is to provide more detailed spatiotemporal information on the chemical processes occurring in the brain that are associated with neurotransmission and its relationship to learning, cognitive behaviour and disease status and progression. The utility of MRI is improved by the use of contrast agents, especially those that enhance specificity and sensitivity by targeting certain regions of interest, or that allow the monitoring of changes in chemical composition as a function of neural activity. This rationale has driven the recent development of contrast agents that selectively target the various glutamate receptors that are found on the cell surface of astrocytes in high abundance. Such responsive contrast agents must be able to bind selectively and reversibly to the receptor, so that changes in local contrast agent levels can be modulated following the glutamate bursts that periodically occur following a stimulus. Given that release of glutamate from a presynaptic cell takes place over a period of milliseconds and should displace the contrast agent from the cell surface receptor, MRI may be able to monitor the restoration to equilibrium that occurs over a period of a second or two. As the glutamate levels drop and the contrast agent competes in binding to the receptor, glutamate is displaced, leading to local signal intensity enhancement once more. Such a perturbational approach requires that the contrast agents bind reversibly and with relatively high affinity to the target site, with minimal non-specific binding.

For these reasons, we have set out to examine conjugates of gadolinium contrast agents with established competitive antagonists for glutamate receptors. When bound to the receptor site, the local rotational dynamics and second sphere of hydration around the gadolinium moiety are perturbed leading to relaxivity enhancement and increased contrast. In our initial work with NMDA receptor targeted gadolinium conjugates, we reported the synthesis and behaviour of competitive NMDAR antagonists based on a 3,4-diamino-3-cyclobutene-1,2-dione developed by Kinney. The NMDA receptor is a tetrameric complex, most commonly built up from alternating GluN1 and GluN2 subunits. Based on the work of Kinney, these receptor binding moieties are a class of competitive antagonists. Therefore, they must bind at the GluN2 subunit where the glutamate binding site is located. However, the GluN2 subunit can be one of four genetic products (GluN2A-D) and the relative expression levels of each can vary between each receptor. In initial work, immunofluorescence staining experiments were undertaken to establish the presence of the GluN2B subunit.

The targeting moieties based on the competitive antagonist moiety were conjugated to a modified Gd-DOTA complex that possesses a single fast-exchanging water molecule, e.g. [Gd.L^ad], Scheme 1. When water exchange at the Gd centre is fast (typically of the order of $10^7$ s$^{-1}$ at 298 K), then the relaxivity, (the increment of the water proton relaxation rate per unit complex concentration) in the low to mid-field range (1 to 3 T), is dominated by rotational dynamics and hence is more sensitive to receptor binding. Initial studies in cell suspensions
of the NSC-34 cell line showed relaxation enhancements of up to 75% on cell surface binding versus controls lacking the NMDA receptors.\textsuperscript{13} The conjugates were non-toxic, as revealed by standard IC\textsubscript{50} studies using an MTT assay that assesses perturbation of mitochondrial redox status, consistent with evidence from optical microscopy studies that revealed no evidence for internalization of the conjugate. Furthermore, the confocal microscopy studies established both the specificity and the reversibility of probe binding, in the absence and presence of added glutamate.\textsuperscript{13}

Here, we report the synthesis and evaluation of an additional series of complexes constituting a second-generation, [Gd.L\textsuperscript{1}-L\textsuperscript{6}] (Scheme 1) in which the monocyclic antagonist group is replaced by a bicyclic moiety, for which receptor binding affinities were established to be about an order of magnitude higher, in the original small molecule work.\textsuperscript{11b} The nature of the spacing chain differs in each case and four examples with shorter linkages (Gd.L\textsuperscript{1}-Gd.L\textsuperscript{4}) were examined together with two analogues (Gd.L\textsuperscript{5}, Gd.L\textsuperscript{6}) in which the spacing chain was longer. The rationale behind this selection of targets was that the further away the targeting vector was from the paramagnetic Gd\textsuperscript{3+} centre, the less likely that receptor binding would be compromised. However, the longer the chain the less motional coupling will occur between the gadolinium moiety and the slowly tumbling macromolecule, comparing free and receptor bound complexes and hence the smaller may be any relaxivity enhancement.

In addition, we have examined the behaviour of the biotin conjugate, [Gd.L\textsuperscript{7}], in which the biotin moiety allows selective tagging to fluorescently labelled avidin conjugates to allow visualization of the avidin-biotin probe conjugate using confocal microscopy.
Results and Discussion

Synthesis of the ligands and complexes

The synthesis of the required bicyclic antagonist moieties followed methods adapted from the work of Kinney.\textsuperscript{11b} Orthogonal protection of 1,3-diamino-propan-2-ol was achieved in high yield following stepwise Boc and $Z$ protection steps.\textsuperscript{14a} Subsequent mesylation of the free alcohol, followed by substitution with NaN$_3$ in DMF afforded the azide, 3 (Scheme 2). The azide group served as a ‘masked’ amine, which was transformed to a primary amine group in the final step of the synthesis. A reagent for selective removal of the CBz protecting group from 3 was required. This ruled out the use of standard deprotection methods, such as hydrogenation over a palladium catalyst, as this would also reduce the azide group. Instead, Ba(OH)$_2$ in refluxing aqueous glyme was used, and gave the free amine, 4, in good yield.\textsuperscript{14b} N-Alkylation with, for example, diethyl-(2-bromo)ethyl phosphonate in the presence of Na$_2$CO$_3$ gave the mono-substituted amine, 7, in modest yield, which was used in a Michael addition-elimination reaction with diethoxy-3-cyclobutene-1,2-dione. The phosphonate ester 11 was obtained as a pair of rotamers in a 1:1.2 ratio as deduced by $^{31}$P NMR spectroscopy. Cyclisation was achieved in high yield after selective hydrolysis of the Boc protecting group, followed by heating the free amine in ethanol in the presence of a tertiary amine. By studying the $^1$H NMR spectrum of the cyclised azide, 15, it was possible to deduce that the conformation adopted by this and related bicyclic intermediates involved selective population of a twist-chair isomer (ESI). In a similar manner, compounds 13, 14 and 16 were prepared.
Scheme 2.  Synthesis of the competitive antagonist moieties (adapted from reference 11b)

With the azide in hand, mild, selective reduction to unmask the primary amine functionality was accomplished using a standard Staudinger reaction, using triphenylphosphine in a THF/H₂O solvent mixture. The reduction proceeded cleanly to yield the primary amines 17-20, as racemic mixtures.
The final step in the synthesis of [Gd.L\textsuperscript{1-6}] involved amide bond formation with the carboxylic acid of a DO3A-glutarate derivative (Scheme 3).\textsuperscript{15} This step was undertaken using EDC/HOBt for the formation of the active ester in the presence of NMM to deprotonate the primary amine. For the phosphonate series, removal of the ethyl ester groups was undertaken using TMS-Br in DMF. Complexation with GdCl\textsubscript{3} proceeded cleanly at pH 6, and the complexes were purified by RP-HPLC following treatment with Chelex resin to remove any excess Gd salts. The overall isolated yields were rather low (20-30%), and gave the paramagnetic complexes as a mixture of diastereoisomers in each case.

The synthesis of the biotin conjugate, [Gd.L\textsuperscript{7}] proceed via a similar reaction sequence (Scheme 4). Z-Deprotection of the differentially protected penta-ester, 23,\textsuperscript{15} followed be reaction with succinic anhydride in DMF afforded the monocarboxylic acid, 24. Following amide coupling (EDC, HOBt, NMM) to the antagonist derivative, 19, hydrolysis of the methyl ester in dilute base enabled coupling of the resultant carboxylic acid with an ethylenediamine derivative of biotin. Stepwise deprotection of the Boc and phosphinate ethyl groups gave the ligand L\textsuperscript{7} and complexation with GdCl\textsubscript{3} proceeded well at pH 6 to afford [Gd.L\textsuperscript{7}]. This complex could be used in confocal microscopy experiments in the presence of a fluorescently labeled avidin host molecule, in order to verify cell surface binding to the NMDA receptors of NSC-34 cells.

**Relaxivity properties and cell suspension MRI studies**

The longitudinal proton relaxivities of the seven Gd complexes were measured in water at 1.4 T and 310 K, and the values fell in the expected range for such mono-aqua complexes of MW 850 to 1450, for which the relaxivity value increases with molecular volume, as it scales with
the overall rotational correlation time. Values were also measured in the presence of human serum albumin (Table 1).

**Table 1.** Estimated binding affinities for serum albumin, a,b,c,d and relaxation properties of the Gd complexes (310 K, pH 7.4, 1.4 T).

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<td>2.2</td>
<td>2.2</td>
<td>1.9</td>
<td>3.7</td>
<td>1.6</td>
<td>1.6</td>
<td>3.7</td>
</tr>
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<td>$r_{lp\text{initial}}^{\text{a,b,c,d}}$/$\text{mM}^{-1}\text{s}^{-1}$</td>
<td>4.91</td>
<td>5.45</td>
<td>5.76</td>
<td>5.21</td>
<td>4.97</td>
<td>5.18</td>
</tr>
<tr>
<td>$r_{lp\text{limit}}^{\text{a,b,c,d}}$/$\text{mM}^{-1}\text{s}^{-1}$</td>
<td>18.0</td>
<td>22.0</td>
<td>7.50</td>
<td>30.0</td>
<td>25.0</td>
<td>8.50</td>
</tr>
<tr>
<td>$r_{lp}/\text{mM}^{-1}\text{s}^{-1}$</td>
<td>5.99</td>
<td>5.42</td>
<td>6.63</td>
<td>5.89</td>
<td>5.51</td>
<td>6.82</td>
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(a) Errors in logK values associated with experimental variation and statistical fitting were estimated to be ± 0.1. The binding constants were estimated assuming a 1:1 stoichiometry; the relative affinity constant (IC50 value) for a structurally similar NMDA binding moiety (the anionic alcohol analogue of the phosphinate) was reported to be 19 nM, determined by a radiolabelled competition assay. b Statistical errors associated with the measurement of $r_{lp}$ values are ±0.03; c the $r_{lp\text{initial}}^{\text{a,b,c,d}}$ and $r_{lp\text{initial}}^{\text{a,b,c,d}}$ values were derived from the iterative least-squares fitting analysis; values for the complexes of L^4 and L^5 are subject to the largest error. d for $[\text{Gd.L}^1]^2^-$, the relaxivity value in water was 7.23 mM$^{-1}$s$^{-1}$ (310 K, 60 MHz)

Human serum albumin (HSA) constitutes around 4.5% of plasma and is the major protein constituent in the circulatory system. Each contrast agent will bind to the protein to some extent, disrupting its rotational dynamics and leading to an enhancement of the longitudinal relaxation rate of the water protons. As a control experiment, the effect of added HSA on the measured relaxivity of $[\text{Gd.L}^1]^2^-$ (each at 1 mM) was assessed at 1.4 T following incremental addition of up to 1.6 mM HSA. Increases in $r_{lp}$ were observed and association constants were estimated by assuming a 1:1 stoichiometry of interaction. No particular trend was evident, correlating logK values with structure; indeed the weakest interaction occurred with the phosphonates, $[\text{Gd.L}^1]^2^-$ and $[\text{Gd.L}^5]^2^-$, whilst the analogues with one less, $[\text{Gd.L}^3]^2^-$, and one more methylene groups, $[\text{Gd.L}^6]^2^-$, bound to albumin 100 times more strongly in each case. At a protein concentration of 0.7 mM, the highest relaxivity values were measured for $[\text{Gd.L}^3]^2^-$ and $[\text{Gd.L}^6]^2^-$, consistent with their higher binding affinities.

A neuronal cell line model that expresses functional NMDA receptors had been established earlier using the NSC-34 cell line, produced by fusing mouse spinal cord and neuroblastoma cells. The cell line has been used in several studies of the evaluation of NMDAR antagonists. The expression of functional NMDA receptors on differentiated NSC-34 cells was demonstrated as reported earlier using immuno-fluorescence techniques with primary antibodies. The labelling of differentiated NSC-34 cells with $[\text{Gd.L}^1]^2^-$ was assessed by measuring the longitudinal relaxation
times, $T_1$, of the water signal in cell suspensions using a Siemens human whole body MR scanner, equipped with a head coil operating at 3 T. These measurements allowed the calculation of cellular relaxation rates, $R_{1,\text{cell}}$. Differentiated cells were incubated for 45 min with 200 µM [Gd.L\textsubscript{1\textperthousand 6}] (37 °C, 5% CO\textsubscript{2}), washed with Hank’s Buffered Saline Solution (HBSS) to remove unbound complex, re-suspended in fresh buffer and $R_1$ was determined in cell suspensions using an inversion recovery sequence (Fig. 1). Values for $R_2$ were also measured.

![Figure 1](image)

**Figure 1** Cellular $^1$H MR relaxation rates, (a) $R_{1,\text{cell}}$ and (b) $R_{2,\text{cell}}$ in cell suspensions (3 T, 298 K) after treatment of differentiated NSC-34 cells with 200 µM [Gd.DOTA]$^\dagger$ or [Gd.L\textsubscript{1\textperthousand 4}] for 45 minutes. Under the same conditions, the Gd$^{3+}$ complexes of LX-L\textsubscript{d} gave corresponding changes of +18, +70, -9 and +76% respectively.$^{13}$ [Gd.DOTA]$^\dagger$ served as a negative control. Values are mean ± SEM (n=4-8). *P<0.05, **P<0.01, ***P<0.001 statistical significant difference vs. untreated control; $^a$ P<0.001 statistical significant difference vs. [Gd.DOTA]$^\dagger$ (ANOVA with Dunnett’s multiple comparison post test, assessed using Graphpad Prism 5.04).

The largest enhancements in relaxation were evident with [Gd.L\textsubscript{5}]$^2$ for which the enhancement was 19% in $R_1$ and 37.5% in $R_2$ over the untreated control and [Gd.DOTA]$^\dagger$ treated cells. Moreover, this was one of the complexes exhibiting the weakest binding to serum albumin (Table 1). Given that albumin binding may be considered as a model for ‘non-specific’ binding to a protein, such behaviour is encouraging. The much lower $R_{1,\text{cell}}$ value for [Gd.L\textsubscript{6}]$^2$, notwithstanding the addition of only a single methylene group to the targeting moiety, accords with the lower receptor affinity of such antagonists in earlier work.$^{11b}$ The set of complexes [Gd.L\textsubscript{1\textperthousand 4}] each gave less than 6 to 12 % enhancements in $R_1$ and $R_2$ respectively, suggesting that the receptor binding moiety may be too close to the bulky Gd complex, leading to inhibition of probe binding to the cell surface receptor, or that local
hydration or water exchange dynamics are compromised for the receptor bound complex in these cases (*vide infra*).

The IC$_{50}$ values of the gadolinium complexes [Gd.L$_{1-6}$] were assessed using an MTT assay that probes the perturbation of mitochondrial redox activity of the differentiated NSC-34 cells.$^{19}$ Following a 24-hour incubation, each complex exhibited no cytotoxic effect at concentrations of up to 200 $\mu$M (data not shown), consistent with the hypothesis that the anionic probes were not being internalized into the cell. This hypothesis was corroborated by measuring the Gd cell uptake using ICP-MS. After a 24h incubation, followed by washing of the cells with fresh buffer three times, less than 2% Gd$^{3+}$ cell uptake was measured in each case, and the gadolinium was found in the washings (ca. 98%).

**Cellular optical imaging**

It was hypothesised that the observed increase in relaxation rates per cell were due to binding of the Gd probe to NMDA receptors on the cell surface. However, cell labelling of the Gd complex via non-specific binding to the cell membrane or by internalisation involving receptor-mediated endocytosis are plausible mechanisms that may lead to the observed increase in $R_{1,\text{cell}}$. To distinguish these possibilities, the biotin complex was designed to allow tracking of the contrast agent on the cell surface.

By attaching a fluorescent label to a non-competitive antagonist, the direct visualisation of the NMDAR-2B subunit has been demonstrated recently.$^{20}$ Accordingly, [Gd.L$_7$] was designed, bearing the antagonist binding moiety of [Gd.L$_5$], as this probe had shown the largest increase in $R_{1,\text{cell}}$ values. The complex includes a remote trans-substituted biotin sub-unit, attached to the macrocyclic core. It was hypothesised that if cell-surface receptor binding is responsible for the increase in $R_{1,\text{cell}}$ for [Gd.L$_5$], the biotin moiety of [Gd.L$_7$], after forming a tightly bound complex to added AvidinAlexaFluor® 488 conjugate, would be tagged to the outside of the cell. The presence of the fluorescent dye on the avidin sub-unit allows direct visualisation of the biotin complex using fluorescence microscopy, only when bound to the cell surface.

In order to study the behaviour and localisation of [Gd.L$_7$], laser scanning confocal microscopy (LSCM) imaging studies were carried out using live cells. The differentiated NSC-34 cells were grown on a microscope slide with a flow-through channel and were subjected to various different experimental conditions. Following incubation of the cells with a solution of [Gd.L$_7$] (10 $\mu$M) and AvidinAlexaFluor®
488 conjugate (2.5 µM, 10 min) together, the cells were examined by microscopy and a localization profile was observed resembling ‘pit-like’ regions at the cell surface (Fig. 2A). The dye CellMask™ Orange is commercially available and can be used to stain the cell plasma membrane non-specifically. By repeating the simultaneous loading experiment of [Gd.L⁷] and AvidinAlexaFluor® 488 conjugate (10 min) and co-incubating with CellMask™ Orange (5 min, 5 µg/mL), unequivocal evidence for the selective localisation of [Gd.L⁷] at the cell membrane was obtained (Fig. 2B/2C). Furthermore, by using the same microscope settings as used previously,¹³ it was possible to demonstrate that cells treated with [Gd.L⁷] were 38% brighter than when they were treated with the acyclic analogue, consistent with the bicyclic targeting moiety having a higher receptor affinity.

Confirmation that the observed fluorescence was due to the strong biotin-avidin interaction between [Gd.L⁷] and AvidinAlexaFluor® 488 conjugate on the cell surface was given through various control experiments. Differentiated cells loaded with AvidinAlexaFluor® 488 conjugate only and differentiated but untreated NSC-34 cells each gave rise to no fluorescence signal in the visible region, using the same experimental parameters as above. Cell surface localisation did not vary for stepwise vs. simultaneous incubations of the biotin and avidin moieties and was independent of time (5 to 45 minutes) and the loading concentration (up to 100 µM). No staining was observed with NIH-3T3 cells (mouse skin fibroblasts) that do not possess the NMDA receptors at the cell surface. In each of these control experiments, no evidence for any significant intracellular staining was observed.
Figure 2. Live cell laser scanning confocal microscopy (LSCM) images of differentiated NSC-34 cells following treatment with [Gd.L₇].

(A) Simultaneous loading of [Gd.L₇] (10 μM), and AvidinAlexaFluor® 488 conjugate (2.5 μM, 10 min) allowing visualisation of AvidinAlexaFluor® 488 conjugate λ_ex/λ_em = 488/505-555 nm.

(B) As in (A), but with a 5 minute incubation of the non-specific cell-surface membrane dye CellMask™ Orange, allowing visualisation of CellMask™ Orange λ_ex/λ_em = 543/550-660 nm. (C) RGB merge showing co-localisation of the AvidinAlexaFluor® 488 conjugate and CellMask™ Orange on the cell surface.

(D) As in image (A) but with a post glutamate (1mM) wash showing that [Gd.L₇] is removed from the cell surface. (E) As image (D) and then simultaneous loading of [Gd.L₇] (10 μM), and AvidinAlexaFluor® 488 conjugate (2.5 μM, 45 min) allows 98% recovery of the fluorescence signal intensity as compared to image (A). (F) Loading of the antagonist, 27, (10 μM, 10 min) followed by simultaneous loading of [Gd.L₇] (10 μM), and AvidinAlexaFluor® 488 conjugate (2.5 μM, 10 min).

(G) Loading of 27 (10 μM, 10 min), followed by a glutamate wash (1 mM) and then simultaneous loading of [Gd.L₇] (10 μM), and AvidinAlexaFluor® 488 conjugate (2.5 μM, 45 min). (H) Simultaneous loading of [Gd.L₇] (10 μM), AvidinAlexaFluor® 488 conjugate (2.5 μM) and [Gd.L₅] (10 μM, 10 min). (I) Simultaneous loading of [Gd.L₇] (10 μM), AvidinAlexaFluor® 488 conjugate (2.5 μM) and [Gd.L₅] (10 μM, 10 min). See also the data given in Table 2.

Competition experiments and reversibility of receptor binding
The reversibility of $[^\text{Gd.L}]$ receptor binding was demonstrated using a glutamate competition experiment. Differentiated NSC-34 cells were incubated with a solution of $[^\text{Gd.L}]$ (10 µM) and AvidinAlexaFluor® 488 conjugate (2.5 µM, 10 min). After washing the cells with five successive aliquots ($V_{tot}=500$ µL) of culture medium that was enriched in glutamate (1 mM), a nine-fold drop in fluorescence intensity was observed, compared to the original cell staining experiment (Fig. 2D). The ability of the probe to displace glutamate was also demonstrated. When differentiated NSC-34 cells were sequentially treated the avidin/biotin components and with five volumetric aliquots of a glutamate rich (1 mM) culture medium, then washed with normal culture media and finally incubated with a solution of $[^\text{Gd.L}]$ (10 µM) and AvidinAlexaFluor® 488 conjugate (2.5 µM, 10 min), 38% of signal was observed after 10 minutes and 98% of the original fluorescence signal intensity was recovered after 45 minutes (Fig. 2E). Such behaviour established the ability of the probe $[^\text{Gd.L}]$ to displace glutamate from the NMDA receptor-binding site, as may occurred after a glutamate burst during neural signalling.

In summary, these results show that $[^\text{Gd.L}]$ is able to bind to the cell surface glutamate-binding site of the NMDAR, via the antagonist entity.

Table 2  Changes in the observed fluorescence intensity of $[^\text{Gd.L}]$-Avidin-Alexa-488 conjugate (2.5 µM), under various conditions.

<table>
<thead>
<tr>
<th>Relative Fluorescence Intensity %</th>
<th>$[^\text{Gd.L}]^a$, post Glu wash$^b$</th>
<th>$[^\text{Gd.L}]^a$, Glu wash$^b$, $[^\text{Gd.L}]^a$ (10/45 mins)</th>
<th>Antagonist, $[^\text{Gd.L}]^a$, Glu wash$^b$, $[^\text{Gd.L}]^a$ (1:1)</th>
<th>Antagonist $[^\text{Gd.L}]^a$ and $[^\text{Gd.L}]^a$ (1:1)</th>
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<tbody>
<tr>
<td>100</td>
<td>11</td>
<td>38/98</td>
<td>&lt;7</td>
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<td>56</td>
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$^a$ Concentration of $[^\text{Gd.L}]$ was 10 µM; $^b$ concentration of added glutamate in the medium was 1 mM; $^c$ concentration of the antagonist 27, was 10 µM in each case; $^d$ concentration of $[^\text{Gd.L}]^{38}$ = 10 µM.

The competitive antagonist, 27, was synthesized in accordance with the methods of
Kinney, and has been shown to bind to the NMDA receptor with an affinity of the order of 20 nM.\textsuperscript{11b} Co-incubation of 27 (10 µM) with [Gd.L\textsuperscript{7}]\textsuperscript{2-} and the Alexa-Fluor avidin conjugate gave rise to <7% of signal intensity (Fig. 2F vs Fig. 2A). By carrying out the following incubation sequence sequentially, first 27, then a Glu wash and finally incubation with [Gd.L\textsuperscript{7}]\textsuperscript{2-}, the observed signal grew to 18%. Evidently, the small molecule has a higher affinity for the NMDA receptor than the Gd probe, [Gd.L\textsuperscript{7}]\textsuperscript{2-}, but the restoration of some observed signal (18%) after the glutamate wash (Fig. 2G) suggests that it may be important not to have a probe that is bound too tightly, or glutamate may not compete for receptor binding.

Finally, competition experiments with equal concentrations of the parent complexes, [Gd.L\textsuperscript{3,5}]\textsuperscript{2-} and [Gd.L\textsuperscript{7}]\textsuperscript{2-} were undertaken (Table 2 and Fig. 2H and I), and the observation of about 50% of the original signal in each case is consistent with each of these complexes having a similar binding affinity for the NMDA receptor site.

Conclusions

A second generation of MR imaging probes has been created that target the NMDAR, based on a competitive antagonist approach. Of the series of six probes examined, [Gd.L\textsuperscript{5}], showed a 19%, increase in the measured water proton relaxation rate in the presence of functional NMDARs on differentiated NSC-34 cells. Cell-surface localisation was demonstrated using confocal microscopy for the biotin-functionalised derivative, [Gd.L\textsuperscript{7}]. No evidence was found for probe internalisation during the time-periods used, neither was any evidence found for cell-surface binding, following probe incubation with an NMDA receptor negative cell-line, consistent with the inherent specificity of this approach.

Binding of the Gd contrast agents to the NMDA receptors was shown to be reversible. Following addition of glutamate to the receptor-bound conjugate [Gd.L\textsuperscript{7}], displacement of the complex from the receptors occurred, leading to a reduction of signal intensity in microscopy, that could be partially recovered by subsequent incubation with [Gd.L\textsuperscript{7}] and the AvidinAlexaFluor\textsuperscript{®} 488 conjugate.

These \textit{in vitro} and \textit{in cellulo} studies suggest that such probes could be used in MRI studies \textit{in vivo}, when the probes are injected intra-cranially to maintain a steady state local concentration, that may allow time-dependent modulation of glutamate concentration to be observed.

Experimental
**Materials and Methods**

All solvents used were laboratory grade and anhydrous solvents, when required, were freshly distilled over the appropriate drying agent. Water was purified by the ‘PuriteSTILLplus’ system, with conductivity of \( \leq 4 \, \mu \text{S cm}^{-1} \). All reagents used were purchased from commercial suppliers (Acros, Aldrich, Fluka, Merck and Strem) and were used without further purification unless otherwise stated. Reactions requiring anhydrous conditions were carried out using Schlenk line techniques under an atmosphere of argon.

Thin layer chromatography was performed on neutral aluminium sheet silica gel plates (Merck Art 5554) and visualised under UV irradiation (254 nm), or using Dragendorff reagent staining. Preparative column chromatography was performed using silica gel (Merck Silica Gel 60, 230-400 mesh).

Reverse phase HPLC was conducted at 298 K using a Shimadzu system. XBridge C18 4.6 x 100 mm, i.d. 5 µm analytical column and XBridge C18 OBD 19 x 100 mm, i.d. 5 µm preparative columns were used to analyse and purify the complexes. A gradient elution with a solvent system composed of \( \text{H}_2\text{O} + 0.1\% \text{HCOOH}/\text{MeOH} + 0.1\% \text{HCOOH} \) was performed for a total run time of 20.0 min.

\(^1\text{H}, \, ^{13}\text{C} \) and \(^{31}\text{P} \) NMR spectra were recorded in commercially available deuterated solvents on a Varian Mercury-400 (\(^1\text{H} 399.960, \, ^{13}\text{C} 100.572\)), Bruker Avance-400 (\(^1\text{H} 400.052, \, ^{13}\text{C} 100.603 \) and \(^{31}\text{P} 161.91\)), Varian Inova-500 (\(^1\text{H} 499.722, \, ^{13}\text{C} 125.671\)), Appleby VNMRS-600 (\(^1\text{H} 599.832, \, ^{13}\text{C} 150.828\)), or Varian VNMRA-700 (\(^1\text{H} 699.731, \, ^{13}\text{C} 175.948 \) and \(^{31}\text{P} 283.26\)) spectrometer. All chemical shifts are given in ppm with coupling constants in Hz.

Low resolution electrospray mass spectra (LR-MS) were recorded on a Fisons VG Platform II, Waters Micromass LCT or Thermo-Finnigan LTQ FT instrument operating in positive or negative ion mode as stated, with MeOH as the carrier solvent. Accurate mass spectra [High resolution electrospray mass spectra (HR-MS)] were recorded using the Thermo-Finnigan LTQ FT mass spectrometer.

Melting points were recorded using a Gallenkamp (Sanyo) apparatus and are uncorrected.

Relaxivity measurements were carried out at 310 K, 60 MHz (1.4 T) on a Bruker Minispec mq60 instrument. The mean value of three independent measurements was recorded. The relaxivities of the compounds were calculated as the slope of the function shown in the equation below,

\[
\frac{1}{T_{1,\text{obs}}} = \frac{1}{T_{1,d}} + r_{L} [\text{Gd.L}^n]
\]

where \( T_{1,\text{obs}} \) is the measured \( T_1 \), \( T_{1,d} \) is the diamagnetic contribution of the solvent (calculated to be 4000 ms) and \([\text{Gd.L}^n] \) is the concentration in mM of the appropriate Gd\(^{3+} \) complex (n = 1 - 6). Errors for all relaxivity values were less than 0.3 mM\(^{-1}\)s\(^{-1}\).
The apparent binding constant for the interaction of the Gd$^{3+}$ complex with Human Serum Albumin (HSA) was calculated using equation (2) below:

\[
[X] = \frac{(R - R_0)/(R_i - R_0)}{K} + [GdL^n]^n \frac{(R - R_0)}{(R_i - R_0)} - [GdL^n]^n \frac{(R - R_0)^2}{(R_i - R_0)}
\]

\[
K = \frac{[GdX]}{[X_f][Gd_f]}
\]

where [X] is the total concentration of HSA in the solution; [Gd.L$^n$]: the total concentration of the complex; K: the binding constant; R: relaxation rate of a given concentration of X; R$_0$: the initial relaxation rate; R$_i$: final relaxation rate; [Gd.X]: the concentration of the HSA-coordinated complex; [X$_f$]: the concentration of free HSA in the mixture; [Gd$_f$]: the concentration of the free complex.

Details of cell experiments, microscopy, and the MTT assays used have been given in earlier papers in this series. $^7$-$^9$,13 The other compounds, ligands and Gd complexes not reported below are described in the ESI.

**Ligand and Complex synthesis**

(3-Amino-2-hydroxypropyl)carbamic acid 1,1-dimethylethyl ester$^{11b}$, 1

A solution of 1,3-diamino-2-hydroxypropane (5.0 g, 55.5 mmol) in anhydrous acetonitrile (50 mL) was maintained at room temperature and treated with a solution of di-tert-butyl dicarbonate (4.04 g, 18.5 mmol) in anhydrous acetonitrile (20 mL) over a 2-hour period with vigorous stirring. The subsequent suspension was left to stir overnight. After a period of 18 hours, the reaction mixture was concentrated and the residue re-dissolved in brine (50 mL). The pH was adjusted to 5 by treatment with HCl (1 N), washed with dichloromethane (3 x 50 mL) and made basic (pH 12) by the addition of NaOH solution (2.5 M). The product was extracted using copious amounts of CHCl$_3$ (5 x 100 mL), dried over MgSO$_4$, filtered and concentrated to yield 1 as a white solid (1.23 g, 34%). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 1.43 (9H, s, C(CH$_3$)$_3$), 2.42-2.72 (3H, br, m), 2.78-2.92 (2H, br, m), 3.01-3.15 (1H, br, m), 3.20-
3.35 (1H, br, m), 3.56-3.72 (1H, br, m), 5.05 (1H, br, s). $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 28.4 (C(CH$_3$)$_3$), 44.1, 44.5 (CH$_2$), 71.0 (COH), 79.5 (C(CH$_3$)$_3$), 156.7 (CO). MS (ES$^+$) m/z 191.1 [M + H]$^+$; C$_8$H$_{19}$N$_2$O$_3$ requires 191.1396; found 191.1392. M. Pt. 78-80 °C [cit.$^{11b}$ 77-79 °C].

**Benzyl tert-butyl(2-hydroxypropane-1,3-diyl)dicarbamate, 2** $^{14a}$

![Benzyl tert-butyl(2-hydroxypropane-1,3-diyl)dicarbamate](image)

To a solution of (3-amino-2-hydroxypropyl)carbamic acid 1,1-dimethylethyl ester (1.28 g, 6.72 mmol) in anhydrous dichloromethane (43 mL) was added benzyl chloroformate (1.05 mL, 7.39 mmol) and diisopropylethylamine (1.29 mL, 7.39 mmol) at 0 °C. The resulting solution was stirred under argon at this temperature for 30 mins before being allowed to warm to room temperature and stirred overnight. After complete consumption of starting materials has been revealed by TLC, the reaction mixture was concentrated and the crude residue purified by column chromatography (DCM/MeOH, 100% to 95:5 using 1% increments $R_f = 0.26$) to give a pale green oil (1.64 g, 75%). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 1.43 (9H, s, C(CH$_3$)$_3$), 3.06-3.35 (4H, m, C(CH$_2$)$_2$), 3.76 (1H, quin, J = 6, C(CH$_3$)$_3$), 5.09 (2H, s, CH$_2$Ph), 5.14 (1H, br, OH), 5.49 (2H, br, NH), 7.29-7.38 (5H, m, Ar-H). $^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 28.5 (C(CH$_3$)$_3$), 43.7, 44.1 (CH$_2$), 67.1 (CH$_2$Ph), 70.9 (COH), 80.1 (C(CH$_3$)$_3$), 128.2, 128.3, 128.7, 136.5 (Ar-C), 157.4, 157.6 (CO). MS (ES$^+$) m/z 347.2 [M+Na]$^+$; C$_{16}$H$_{24}$N$_2$O$_5$Na requires 347.1583; found 347.1593.

**11,11-Dimethyl-3,9-dioxo-1-phenyl-2,10-dioxa-4,8-diazadodecan-6-yl methylsulfonate**

![11,11-Dimethyl-3,9-dioxo-1-phenyl-2,10-dioxa-4,8-diazadodecan-6-yl methylsulfonate](image)

Benzyl tert-butyl(2-hydroxypropane-1,3-diyl)dicarbamate (907 mg, 2.80 mmol) was dissolved in anhydrous dichloromethane (16 mL), to which triethylamine (430 µL, 3.08 mmol) and methanesulfonyl chloride (238 µL, 3.08 mmol) were added at 0 °C. The resulting solution was stirred at this temperature for 5 minutes before being warmed to room temperature for a further 1 hour until complete conversion of the starting material was observed by ESI-MS. The solvent was removed under reduced pressure and the residue partitioned between brine and DCM (30 mL, 1:1). The organic portion was separated and the aqueous layer extracted with dichloromethane (3 x 30 mL). The combined organic portions
were dried over MgSO$_4$, filtered and reduced to give a colourless oil which was used directly in the next step without further purification (1.20 g, 100%). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 1.43 (9H, s, C(CH$_3$)$_3$), 3.04 (3H, s, CH$_3$), 3.22-3.59 (4H, m, CH$_2$), 4.68 (1H, br, NH), 5.10 (2H, s, CH$_2$Ph), 5.13 (1H, m, CH), 5.55 (1H, br, NH), 7.31-7.38 (5H, m, Ar-H). MS (ES$^+$) m/z 403.1 [M+H]$^+$. 

**Benzyl tert-butyl (2-azidopropane-1,3-diyl)dicarbamate, 3**

![Chemical Structure](image)  

To a solution of the mesylate (1.20 g, 2.93 mmol) in anhydrous dimethylformamide (13 mL) was added NaN$_3$ (580 mg, 8.93 mmol) at room temperature. After addition, the cloudy mixture was heated to 60 °C under a stream of argon for 20 hours. Once no further conversion of starting materials has been revealed by ESI-MS, the mixture was cooled and the solvent removed under reduced pressure. The residue was partitioned between EtOAc/H$_2$O (40 mL, 1:1) and the organic portion separated. The aqueous was extracted with EtOAc (3 x 40 mL) and the combined organic portions dried over MgSO$_4$, filtered, concentrated to give the crude residue which was purified by column chromatography (hexane/EtOAc, 90:10 to 70:30 using 5% increments; $R_f = 0.51$) to give a colourless oil (605 mg, 59%). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 1.44 (9H, s, C(CH$_3$)$_3$), 3.07-3.28 (2H, m, CH$_2$), 3.31-3.49 (2H, m, CH$_2$), 3.66 (1H, quin, J = 6, CH), 5.04 (1H, br, NH), 5.11 (2H, s, CH$_2$Ph), 5.46 (1H, br, NH), 7.28-7.39 (5H, m, Ar-H). $^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 28.4 (C(CH$_3$)$_3$), 40.9, 41.3 (CH$_2$), 60.9 (CN$_3$), 67.2 (CH$_2$Ph), 80.2 (C(CH$_3$)$_3$), 128.2, 128.4, 128.7, 136.4 (C-Ar), 156.4, 156.9 (CO). MS (ES$^+$) m/z 372.1 [M+Na]$^+$; C$_{16}$H$_{23}$N$_5$O$_4$Na requires 372.1648; found 372.1626.

**tert-Butyl (3-amino-2-azidopropyl)carbamate, 4**

![Chemical Structure](image)  

Benzyl tert-butyl (2-azidopropane-1,3-diyl)dicarbamate (605 mg, 1.73 mmol) was dissolved in anhydrous glyme (40 mL) to which a solution of Ba(OH)$_2$ in H$_2$O (0.15 M, 20 mL) was added. The resulting solution was heated to 80 °C under argon, and the reaction progress monitored by ESI-MS and TLC. After 48 hours, the reaction was cooled to room temperature
and the solvent removed under reduced pressure. The residue was partially dissolved in brine and the pH adjusted to 5 using HCl (1 N). The aqueous portion was washed with dichloromethane (2 x 100 mL) before adjusting the pH to 12 using NaOH solution (2.5 M). The product was extracted with copious amounts of CHCl₃ (5 x 100 mL), dried over MgSO₄, filtered and concentrated to give a colourless oil (257 mg, 69%).

\[ \text{1H NMR (400 MHz, CDCl}_3 \text{)} \delta 1.45 (9H, s, C(CH}_3)_3, 1.56 (2H, bs, s), 2.76 (1H, dd, J = 13, 7, CHHNH), 2.88 (1H, dd, J = 13, 5, CHHNH), 3.20 (1H, dt, J = 13, 7, CHHNH), 3.35 (1H, dt, J = 13, 5 CHHNH), 3.50-3.60 (1H, m, CH), 4.89 (1H, br, NH). \]

\[ \text{13C NMR (101 MHz, CDCl}_3 \text{)} \delta 28.5 (C((C}_3H}_3)_3), 42.1, 43.4 (C}_2H}_2, 64.7 (CH), 78.4 (C(CH}_3)_3), 156.6 (CO). \]

MS (ES⁺) m/z 216.1 [M+H]⁺; C₈H₁₈N₂O₂ requires 216.1461; found 216.1458.

**Methyl 3-((2-azido-3-((tert-butoxycarbonyl)amino)propyl)amino)propanoate, 6**

\[
\text{O} \quad \text{N} \quad \text{N}_3 \\
\text{O} \quad \text{C} \quad \text{C}
\]

A solution of tert-butyl (3-amino-2-azidopropyl)carbamate (200 mg, 0.93 mmol), diisopropylethylamine (243 µL, 1.4 mmol) and methyl-3-bromopropionate (102 µL, 0.93 mmol) in anhydrous DMF (8 mL) was stirred at room temperature for 48 hours until no further reaction was observed by TLC. The mixture was concentrated under reduced pressure before being partitioned between EtOAc/H₂O (1:1, 30 mL). The organic portion was separated and the aqueous extracted with EtOAc (3 x 25 mL). The combined organic layer were dried over MgSO₄, filtered and concentrated, allowing the crude residue to be purified by column chromatography (DCM/MeOH, 100% to 94:6 using 1% increments; \( R_t = 0.47 \)) to give a pale yellow oil (220 mg, 79%).

\[ \text{1H NMR (400 MHz, CDCl}_3 \text{)} \delta 1.43 (9H, s, C(CH}_3)_3, 1.50 (1H, br, NH), 2.77 (2H, dd, J = 12, 7, CH₂), 2.89 (2H, dd, J = 12, 5, CH₂), 3.09 (2H, t, J = 6, CH₂), 3.36 (2H, t, J = 6, CH₂), 3.71 (3H, s, CH₃), 4.02 (1H, m, CH), 5.19 (1H, br, NH). \]

\[ \text{13C NMR (101 MHz, CDCl}_3 \text{)} \delta 28.4 (C(CH}_3)_3), 32.5, 42.0, 44.8, 49.5 (CH₂), 52.3 (CH₂), 59.6 (CN), 80.4 (C(CH}_3)_3), 156.5, 172.4 (CO). \]

MS (ES⁺) m/z 302.2 [M+H]⁺; C₁₂H₂₄N₅O₄ requires 302.1828; found 302.1831.

**Methyl 3-((2-azido-3-((tert-butoxycarbonyl)amino)propyl)(2-ethoxy-3,4-dioxocyclobut-1-en-1-yl)amino)propanoate, 10**
To a solution of 3,4-diethoxy-3-cyclobutene-1,2-dione (102 µL, 0.68 mmol) in anhydrous ethanol (1 mL), was added a solution of methyl 3-((2-azido-3-((tert-butoxycarbonyl)amino)propyl)amino)propanoate (206 mg, 0.68 mmol) in anhydrous ethanol (5 mL) over 30 mins. The resulting solution was stirred under argon at room temperature and the progress of the reaction followed by TLC. When no further reaction was observed by TLC, the solution was concentrated under reduced pressure. The crude residue was then purified by column chromatography (DCM/MeOH, 100% to 95:5 using 0.5% increments; \( R_f = 0.59 \)) to give a colourless oil, (234 mg, 81%). This compound exists as a pair of diastereoisomers. \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 1.41 (9H, s, C(CH\(_3\))\(_3\)), 1.43 (3H, t, J = 7, OCH\(_2\)CH\(_3\)), 2.66 (2H, t, J = 7, CH\(_2\)CH\(_2\)CO\(_2\)Me), 3.08-3.57 (3H, m, CH\(_2\)), 3.66 (3H, s, CH\(_3\)) 3.71-4.07 (4H, m, CH\(_2\)CH), 4.75 (2H, q, J = 7, OCH\(_2\)CH\(_3\)), 5.09 (1H, br, NH). \(^{13}\)C NMR (101 MHz, CDCl\(_3\)) \(\delta\) 15.9 (OCH\(_2\)CH\(_3\)), 28.4 (C(CH\(_3\))\(_3\)), 33.9, 42.2, 46.2, 51.1 (CH\(_2\)), 52.1 (CH\(_3\)), 61.2 (CN\(_3\)), 70.1 (OCH\(_2\)CH\(_3\)), 80.1 (C(CH\(_3\))\(_3\)), 155.9 (CO), 171.2 (C=O), 172.7 (CO), 177.4 (C=C), 183.0, 188.4 (CO). MS (ES\(^+\)) \(m/z\) 851.1 [2M+H]\(^+\); \(\text{C}_{36}\text{H}_{55}\text{N}_{10}\text{O}_{14}\) requires 851.3899; found 851.3918.

**Methyl 3-(4-azido-8,9-dioxo-2,6-diazabicyclo[5.2.0]non-1(7)-en-2-yl)propanoate, 14**

To a solution of methyl 3-((2-azido-3-((tert-butoxycarbonyl)amino)propyl)(2-ethoxy-3,4-dioxocyclobut-1-en-1-yl)amino)propanoate (78 mg, 0.18 mmol) in anhydrous dichloromethane (1 mL) was added TFA (1 mL), and the resulting solution stirred at room temperature for 1 hour. Reaction was complete after this period, as indicated by TLC, and the solvents were removed under reduced pressure. The residue was re-dissolved in dichloromethane and again concentrated under reduced pressure. This process was repeated 5 times to ensure complete removal of excess TFA. The TFA salt was dissolved in anhydrous ethanol (2 mL) to which a solution of triethylamine (102 µL, 0.736 mmol) in anhydrous
ethanol (1.5 mL) was added over a 20 minute period. The resulting solution was heated to reflux for 18 hours, until no further reaction was observed by TLC. At this point, the solvent was removed and the crude residue purified by column chromatography (DCM/MeOH, 100% to 90:10 using 1% increments; R_t = 0.52) to give a colourless oil (42 mg, 82%).  

1H NMR (700 MHz, DMSO-d_6) δ 2.69 (2H, overlapping dt, J = 14, 7, CH_2CO_2Me), 3.40 (1H, dd, J = 14, 7, H_a(eq)), 3.55 (1H, ddd, J = 14, 7, 4, H_a(axial)), 3.57 - 3.59 (2H, br, m, H_c(ax/eq)), 3.60 (3H, s, CH_3), 3.86 (1H, dt, J = 14, 7, CH_2CH_2CO_2Me), 3.94 (1H, dt, J = 14, 7, CH_2CH_2CO_2Me), 4.31 (1H, m, CH), 8.53 (1H, br, NH).

13C NMR (176 MHz, DMSO-d_6) δ 33.0 (C_e), 45.9 (C_H_2CH_2CO_2Me), 47.5 (C_a), 51.5 (CH_3), 54.4 (CH_2CH_2CO_2Me), 58.9 (CN), 167.7, 168.3 (C=C), 171.1, 180.9, 181.0 (CO). MS (ES^+) m/z 280.1 [M+H]^+; C_{11}H_{14}N_5O_4 requires 280.1046; found 280.1067.

Methyl 3-(4-amino-8,9-dioxo-2,6-diazabicyclo[5.2.0]non-1(7)-en-2-yl)propanoate, 18

To a solution of methyl 3-(4-azido-8,9-dioxo-2,6-diazabicyclo[5.2.0]non-1(7)-en-2-yl)propanoate (38 mg, 0.14 mmol) in anhydrous THF (3 mL) was added H_2O (100 µL) and PPh_3 (54 mg, 0.20 mmol). The suspension was stirred under argon at 60 °C and over time, became a clear solution. After stirring for 16 hours, the solvent was removed under reduced pressure and the crude residue partitioned between DCM and H_2O (10 mL, 1:1). The aqueous layer was separated and washed twice with DCM (10 mL), before lyophilisation yielded a white solid (34 mg, 99%), m.p. > 250 °C. 1H NMR (700 MHz, DMSO-d_6) δ 1.78 - 2.18 (2H, br, NH_2), 2.69 (2H, overlapping dt, J = 14, 7, CH_2CO_2Me), 3.11 (2H, m, H^6), 3.21 (1H, dd, J = 14, 7, H^6(axial)), 3.27-3.31 (1H, m, H^6), 3.36 (1H, d, J = 14, H^6(eq)), 3.59 (3H, s, CH_3), 3.87 (1H, dt, J = 14, 7, CH_2CH_2CO_2Me), 3.92 (1H, dt, J = 14, 7, CH_2^d), 8.53 (1H, br, NH).

13C NMR (176 MHz, DMSO-d_6) δ 32.9 (CH_2CH_2CO_2Me), 46.2 (CH_2CH_2CO_2Me), 51.5 (CH_3), 51.6 (CH), 51.8 (CH_2), 58.8 (CH_2), 167.3, 168.0 (C=C), 171.3, 180.1, 181.2 (CO). MS (ES^+) m/z 254.1 [M+H]^+; C_{11}H_{16}N_3O_4 requires 254.1141; found 254.1166.

tert-Butyl (2-azido-3-((3-(diethoxyphosphoryl)propyl)amino)propyl)carbamate, 8
A solution of tert-butyl (3-amino-2-azidopropyl)carbamate (395 mg, 1.84 mmol), sodium carbonate (293 mg, 2.76 mmol) and diethyl-3-bromopropyl phosphonate (560 µL, 2.91 mmol) in anhydrous ethanol (8 mL) was boiled under reflux for 16 hours until no further reaction was observed by TLC. The mixture was concentrated under reduced pressure before being partitioned between DCM/H₂O (1:1, 30 mL). The organic portion was separated and the aqueous extracted with dichloromethane (3 x 25 mL). The combined organic fractions were dried over MgSO₄, filtered and concentrated, allowing the crude residue to be purified by column chromatography (DCM/MeOH, 100% to 92:8 using 1% increments; Rf = 0.35) to give the ester as a colourless oil (396 mg, 55%).

**1H NMR (400 MHz, CDCl₃) δ 1.31 (6H, t, J = 7, P(OCH₂CH₃)₂), 1.43 (9H, s, C(CH₃)₃), 1.71-1.86 (5H, m, PCH₂CH₂CH₂ + NH), 2.66 (1H, dd, J = 12, 7, CHH), 2.69 (2H, m, PCH₂CH₂CH₂), 2.71 (1H, dd, J = 12, 5, CHH), 3.17 (1H, dt, J = 14, 5, CHH), 3.63-3.72 (1H, m, CH), 4.02-4.15 (4H, qd, 3Jₕ-H = 7, 3J_H-P = 3, P(OCH₂CH₃)₂), 5.01 (1H, br, NH).**

**13C NMR (101 MHz, CDCl₃) δ 16.6 (d, 3J = 6, P(OCH₂CH₃)₂), 23.0 (d, J = 5, NCH₂CH₂CH₂P), 23.4 (d, 1J = 143, NHCH₂CH₂P), 28.5 (C(CH₃)₃), 42.6 (CH₂), 50.0 (d, 2J = 18, NCH₂CH₂CH₂P), 50.8 (CH₂), 61.7 (d, 2J = 7, P(OCH₂CH₃)₂), 61.9 (CN₃), 79.9 (C(CH₃)₃), 156.1 (CO).**

**31P NMR (CDCl₃, 162 MHz) δ 32.01.**

**MS (ES⁺) m/z 394.2 [M+H]⁺; C₁₅H₃₂N₅O₅P requires 394.2219; found 394.2215.**

**tert-Butyl (2-azido-3-((3-(diethoxyphosphoryl)propyl)(2-ethoxy-3,4-dioxocyclobut-1-en-1-yl)amino)propyl)carbamate, 12**

To a solution of 3,4-diethoxy-3-cyclobutene-1,2-dione (152 µL, 1.03 mmol) in anhydrous ethanol (1 mL), was added a solution of tert-butyl (2-azido-3-((3-(diethoxyphosphoryl)propyl)amino)propyl)carbamate (396 mg, 1.0 mmol) in anhydrous...
ethanol (7 mL) over 30 mins. The resulting solution was stirred under argon at room temperature and the progress of the reaction followed by TLC. After no further reaction was observed by TLC, the solution was concentrated under reduced pressure. The crude residue was then purified by column chromatography (DCM/MeOH, 100% to 95:5 using 0.5% increments; \( R_f = 0.44 \)) to give a colourless oil, existing as a pair of rotamers (417 mg, 79%).

\[^1\text{H} \text{NMR (700 MHz, CDCl}_3\) \delta 1.32 (6H, t, J = 7, P(OCH}_2\text{CH}_3)_2\), 1.44 (9H, s, C(CH}_3)_3\), 1.46 (3H, t, J = 7, OCH}_2\text{CH}_3\), 1.65-1.96 (4H, m, PCH}_2\text{CH}_2\text{CH}_3\), 3.11-3.42 (2H, m, CH}_2\), 3.48-3.89 (5H, m, C(CH}_2)_2+P(CH}_2)_2\), 4.03-4.15 (4H, m, P(OCH}_2\text{CH}_3)_2\), 4.75-4.80 (2H, m, OCCH}_2\text{CH}_3\), 5.04 (1H, br, NH). \[^{13}\text{C} \text{NMR (175 MHz, CDCl}_3\) \delta 16.0 (OCH}_2\text{CH}_3\), 16.6 (d, \(^3J = 6\, \text{Hz}, P(OCH}_2\text{CH}_3)_2\), 22.3 (d, \(^2J = 5\, \text{Hz}, \text{NCH}_2\text{CH}_2\text{CH}_2\text{P}\), 22.6 (d, \(^1J = 143\) \text{Hz}, \text{NCH}_2\text{CH}_2\text{CH}_2\text{P}\), 28.4 (C(CH}_3)_3\), 42.2 (CH}_2\), 50.6 (d, \(^3J = 18\) \text{Hz}, \text{NCH}_2\text{CH}_2\text{CH}_2\text{P}\), 50.8 (CH}_2\), 61.1 (CNi), 62.0 (d, \(^2J = 7\, \text{Hz}, P(OCH}_2\text{CH}_3)_2\), 70.2 (OCH}_2\text{CH}_3\), 80.3 (C(CH}_3)_3\), 156.0 (CO), 172.7, 177.2 (C=C), 182.9, 188.5 (CO). \[^{31}\text{P} \text{NMR (CDCl}_3\, \text{283 MHz)} \delta 30.14. \text{MS (ES}})^+ m/z 518.1 [M+H]^+; \text{C}_{21}\text{H}_{37}\text{N}_{5}\text{O}_{8}\text{P \text{requires 518.2380; found 518.2375.}}

**Diethyl-3-(4-azido-8,9-dioxo-2,6-diazabicyclo[5.2.0]non-1(7)-en-2-yl)propyl)phosphonate, 16**

![Diethyl-3-(4-azido-8,9-dioxo-2,6-diazabicyclo[5.2.0]non-1(7)-en-2-yl)propyl)phosphonate, 16](image)

To a solution of tert-butyl (2-azido-3-((3-(diethoxyphosphoryl)propyl)(2-ethoxy-3,4-dioxocyclobut-1-en-1-yl)amino)propyl)carbamate (201 mg, 0.39 mmol) in anhydrous dichloromethane (1 mL) was added TFA (1 mL), and the resulting solution stirred at room temperature for 1 hour. Reaction was complete after this period, as indicated by TLC, and the solvents were removed under reduced pressure. The residue was re-dissolved in dichloromethane and again reduced under reduced pressure. This process was repeated 5 times to ensure complete removal of excess TFA. The TFA salt was dissolved in anhydrous ethanol (5 mL) to which a solution of triethylamine (218 µL, 1.56 mmol) in anhydrous ethanol (2 mL) was added over a 20 minute period. The resulting solution was heated to reflux for 18 hours, until no further reaction was observed by TLC. At this point, the solvent was removed under reduced pressure and the crude residue purified by column chromatography (DCM/MeOH, 100% to 90:10 using 1% increments; \( R_f = 0.26 \)) to give a colourless oil (126 mg, 87%). \[^1\text{H} \text{NMR (400 MHz, CDCl}_3\) \delta 1.30 (6H, t, J = 7, P(OCH}_2\text{CH}_3)_2\), 1.69-2.01 (4H, m, PCH}_2\text{CH}_2\text{CH}_3\), 3.49 (1H, dd, J = 13, 7, H^a(axial)), 3.52-3.62 (3H, m, H^b(eq), H^b(axial)), 3.71-4.11 (7H, m, CH + NCH}_2\text{CH}_2\text{CH}_2\text{P + P(OCH}_2\text{CH}_3)_2\),
8.16 (1H, br, s, NH). $^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 16.5 (d, $^3J = 6$, P(OCH$_2$CH$_3$)$_2$), 21.8 (d, $^3J = 5$, NCH$_2$CH$_2$CH$_3$P), 22.2 (d, $^3J = 143$, NCH$_2$CH$_2$CH$_3$P), 48.5 (CH$_2$), 51.3 (d, $^3J = 18$, NCH$_2$CH$_2$CH$_3$P), 55.3 (CH$_2$), 59.5 (CN$_3$), 62.0 (d, $^3J = 7$, P(OCH$_2$CH$_3$)$_2$), 167.6, 168.6 (C=C), 180.9, 182.2 (CO). $^{31}$P NMR (CDCl$_3$, 162 MHz) $\delta$ 30.8. MS (ES$^+$) $m/z$ 372.1 [M+H$^+$]; C$_{14}$H$_{23}$N$_5$O$_5$P requires 372.1437; found 372.1436.

Diethyl-3-(4-amino-8,9-dioxo-2,6-diazabicyclo[5.2.0]non-1(7)-en-2-yl)propyl)phosphonate, 20

To a solution of diethyl (3-(4-azido-8,9-dioxo-2,6-diazabicyclo[5.2.0]non-1(7)-en-2-yl)propyl)phosphonate (179 mg, 0.48 mmol) in anhydrous THF (10 mL) was added H$_2$O (250 µL) and PPh$_3$ (189 mg, 0.72 mmol). The suspension was stirred under argon at 60 °C and over time, became a clear solution. After stirring for 16 hours, the solvent was removed under reduced pressure and the crude residue partitioned between DCM and H$_2$O (20 mL, 1:1). The aqueous layer was separated and washed twice with DCM (10 mL), before lyophilisation yielded a white solid (155 mg, 94%), m.p. >250°C. $^1$H NMR (700 MHz, DMSO-$d_6$) $\delta$ 1.23 (6H, t, $J = 7$, P(OCH$_2$CH$_3$)$_2$), 1.72-1.81 (4H, m, PCH$_2$CH$_2$CH$_3$), 3.23-3.38 (2H, br, NH$_2$), 3.39-3.77 (7H, m, CH$_2$ + CH), 3.95-4.01 (4H, qd, $^3J_{H-H} = 7$, $^3J_{H-P} = 3$, P(OCH$_2$CH$_3$)$_2$), 8.57 (1H, s, NH). $^{13}$C NMR (176 MHz, DMSO-$d_6$) $\delta$ 16.3 (d, $^3J = 6$, P(OCH$_2$CH$_3$)$_2$), 21.3 (d, $^3J = 5$, NCH$_2$CH$_2$CH$_3$P), 21.5 (d, $^3J = 143$, NCH$_2$CH$_2$CH$_3$P), 47.9, 50.3 (CH$_2$), 50.7 (d, $^3J = 18$, NCH$_2$CH$_2$CH$_3$P), 54.8 (CH$_2$), 61.0 (d, $^2J = 7$, P(OCH$_2$CH$_3$)$_2$), 167.9, 168.4 (C=C), 181.2, 181.4 (CO). $^{31}$P NMR (162 MHz, DMSO-$d_6$) $\delta$ 31.27. MS (ES$^+$) $m/z$ 346.1 [M+H$^+$]; C$_{14}$H$_{23}$N$_5$O$_5$P requires 346.1532; found 346.1550.

(R)-5-tert-Butoxy-5-oxo-4-[4,7,10-tris(2-tert-butoxy-2-oxoethyl)-1,4,7,10-tetraazacyclododecan-1-yl]pentanoic acid $^{15}$ 21
A solution of \((R)-5\)-benzyl 1-\textit{tert}-butyl 2-[4,7,10-tris(\textit{tert}-butoxy)-2-oxoethyl]-1,4,7,10-tetraazaacyclododecan-1-yl]pentanedioate\(^3\) (677 mg, 0.86 mmol) and Pd-C (10\%) in MeOH (10 mL), was agitated at room temperature, under H\(_2\) (40 psi), in a Parr apparatus for 6 h. The reaction mixture was filtered through Celite and the filtrate evaporated under reduced pressure, to give the acid as a hygroscopic solid (530 mg, 88\%). \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 1.27 (9H, s, C(CH\(_3\))\(_3\)), 1.28 (18H, s, C(CH\(_3\))\(_3\)), 1.29 (9H, s, C(CH\(_3\))\(_3\)), 1.86-2.16 (4H, m, CH\(_2\)CH\(_2\)CO), 2.18-3.18 (16H, br. m, CH\(_2\)), 3.23 (4H, s, COCH\(_2\)), 3.24 (2H, s, COCH\(_2\)), 3.27-3.35 (1H, m, COCH\(_2\)), 3.67 (1H, s, OH). \(^{13}\)C NMR (101 MHz, CDCl\(_3\)) \(\delta\) 27.6, 27.7, 27.9, 28.0 (C(CH\(_3\))\(_3\)), 33.7, 49.7, 52.5, 55.3, 55.6, 56.0, 60.1 (CH\(_2\)), 69.6 (CH), 81.7, 82.0, 82.5 (C(CH\(_3\))\(_3\)), 171.2, 172.6, 175.2, 176.0 (CO). MS (ES\(^{+}\)): \(m/z\) 701.1 [M+H]\(^{+}\); C\(_{35}\)H\(_{65}\)N\(_4\)O\(_{10}\) requires 701.4701; found 701.4704.

\((R)-4-((6-\textit{tert}-Butoxy)-6-oxo-5-(4,7,10-tris(\textit{tert}-butoxy)-2-oxoethyl)-1,4,7,10-tetraazaacyclododecan-1-yl)hexyl)amino)-4-oxobutanoic acid, 22

To a pre-stirred solution of \((R)-tri-\textit{tert}-butyl 2,2',2''-(10-(6-amino-1-(\textit{tert}-butoxy)-1-oxohexan-2-y1)-1,4,7,10-tetraazaacyclododecane-1,4,7-triy1)triacetate\(^7\) (325 mg, 0.46 mmol) and diisopropylethylamine (160 \(\mu\)L, 0.92 mmol) in anhydrous DMF (3 mL), was added succinic anhydride (46 mg, 0.46 mmol) and the resulting solution stirred at room temperature and the reaction monitored by ESI-MS. After 16 hours, the solvent was removed under reduced pressure and the crude residue purified by column chromatography (DCM/MeOH, 100\% DCM to 87:13; \(R_f = 0.32\)) to give a yellow gum (228 mg, 62\%). \(^1\)H NMR (600 MHz, CDCl\(_3\)) \(\delta\) 1.34 (9H, s, C(CH\(_3\))\(_3\)), 1.35 (18H, s, C(CH\(_3\))\(_3\)), 1.36 (9H, s, C(CH\(_3\))\(_3\)), 1.41-1.64
(6H, m), 1.96-2.06 (3H, m), 2.11-2.27 (4H, m), 2.37-2.54 (7H, m), 2.65-2.80 (6H, m), 2.83-2.96 (3H, m), 3.10-3.33 (1H, m), 8.27-8.33 (1H, br. s, OH), 8.85 (1H, br.s, NH). \(^{13}\)C NMR (151 MHz, CDCl\(_3\)) \(\delta \) 24.5 (CH\(_2\)), 26.5, 27.7, 27.8 (C(CH\(_3\))\(_3\)), 28.8, 30.0, 31.8, 39.1, 44.5, 47.1, 48.0, 48.3, 48.4, 52.4, 52.5, 53.5, 55.4, 55.6, 55.7 (CH\(_2\)), 61.1 (CH), 81.9, 82.0, 82.0 (C(CH\(_3\))\(_3\)), 172.5, 172.7, 174.4, 174.8, 175.1 (CO).

MS (ES\(^+\)) m/z 800.4 [M+H]\(^+\); C\(_{40}\)H\(_{74}\)N\(_5\)O\(_{11}\) requires 800.5385; found 800.5366.

[Conjugate 2]

(R)-5-tert-Butoxy-5-oxo-4-[4,7,10-tris(2-tert-butoxy-2-oxoethyl)-1,4,7,10-tetraazaacyclododecan-1-yl]pentanoic acid (94 mg, 0.13 mmol), EDC (31 mg, 0.16 mmol) and HOBt (22 mg, 0.16 mmol) were dissolved in anhydrous DMF (2 mL) and stirred at room temperature under an atmosphere of argon for 20 minutes. After this period, a pre-stirred solution of methyl 3-(4-amino-8,9-dioxo-2,6-diazabicyclo[5.2.0]non-1(7)-en-2-yl)propanoate (34 mg, 0.13 mmol) and NMM (30 \(\mu\)L, 0.27 mmol) in anhydrous DMF (1.5 mL) was added dropwise and the resulting solution stirred at room temperature until complete consumption of the starting materials was revealed by ESI-MS. After this period, the solvent was removed under reduced pressure and the crude oil taken up into EtOAc (20 mL). NaHCO\(_3\) (20 mL) was added, the layers separated and the aqueous fraction washed with EtOAc (3 x 40 mL). The combined organic portions were dried over MgSO\(_4\), filtered and the solvent removed under reduced pressure. The crude residue was purified by column chromatography (DCM/MeOH, 100% to 90:10 in 1% increments; \(R_f \approx 0.37\)) to yield a pale brown viscous oil. This product was characterized as a pair of diastereoisomers (33 mg, 26%). \(^1\)H NMR (600 MHz, CDCl\(_3\)) \(\delta \) 1.42-1.44 (36H, overlapping s, C(CH\(_3\))\(_3\)), 1.82-1.93 (1H, m), 1.95-2.11 (4H, m), 2.17-2.27 (3H, m), 2.34-2.41 (1H, m), 2.49-2.58 (4H, m), 2.60-2.70 (5H, m, CH\(_2\)\(^e\) + CH\(_2\)), 2.71-2.78 (2H, m), 2.78-2.85 (2H, m), 2.89-2.99 (3H, m), 3.29-3.38 (2H, m), 3.40-3.52 (4H, m, H\(^d\) + CH), 3.64 (3H, s, CH\(_3\)), 3.77-3.87 (2H, m, H\(^e\)), 3.94 (1H, dt, J = 14, 7, CH\(_2\)\(^d\)), 4.03 (1H, dt, J = 14, 7, CH\(_2\)\(^d\)), 4.13-4.19 (1H, m, CHNH), 7.72 (1H, br, NH), 8.70 (1H, br, NH). \(^{13}\)C NMR
(151 MHz, CDCl$_3$) $\delta$ 27.9, 28.0 (overlapping C(CH$_3$)$_3$), 33.8 (C$^5$), 35.3, 45.6, 47.2 (C$^6$), 48.2, 48.7, 49.8, 50.2 (CHNH), 51.2 (CH$_3$), 52.8, 55.6, 55.9, 56.4, 60.8 (CH), 82.0, 82.1, 82.1, 82.2 (C(CH$_3$)$_3$), 167.7, 167.9 (C=C), 171.4, 172.6, 172.8, 172.9, 173.5, 175.3, 181.2, 182.0 (CO).

MS (ES$^+$) $m/z$ 936.6 [M+H]$^+$; C$_{46}$H$_{78}$N$_7$O$_{13}$ requires 936.5658; found 936.5672.

[Conjugate 4]

(R)-5-tert-Butoxy-5-oxo-4-[4,7,10-tris(2-tert-butoxy-2-oxoethyl)-1,4,7,10-tetraazacyclodecan-1-yl]pentanoic acid (138 mg, 0.20 mmol), EDC (45 mg, 0.24 mmol) and HOBt (32 mg, 0.24 mmol) were dissolved in anhydrous DMF (3 mL) and stirred at room temperature under an atmosphere of argon for 20 minutes. After this period, a pre-stirred solution of diethyl (3-(4-amino-8,9-dioxo-2,6-diazabicyclo[5.2.0]non-1(7)-en-2-yl)propyl)phosphonate (68 mg, 0.20 mmol) and NMM (43 µL, 0.39 mmol) in anhydrous DMF (2 mL) was added dropwise and the resulting solution stirred at room temperature until complete consumption of the starting materials was revealed by ESI-MS. After this period, the solvent was removed under reduced pressure and the crude oil taken up into EtOAc (30 mL). NaHCO$_3$ (30 mL) was added, the layers separated and the aqueous washed with EtOAc (3 x 50 mL). The combined organic portions were dried over MgSO$_4$, filtered and the solvent removed under reduced pressure. The crude residue was purified by column chromatography (DCM/MeOH, 100% to 90:10 in 1% increments; $R_f$ = 0.41) to yield a dark yellow viscous oil.

This product was characterized as a pair of diastereoisomers (57 mg, 28%). $^1$H NMR (700 MHz, CDCl$_3$) $\delta$ 1.27 (6H, t, $J = 7$, P(OCH$_2$C$_2$H$_5$)$_2$), 1.40, 1.40, 1.41, 1.42 (36H, s, C(CH$_3$)$_3$), 1.66-1.74 (2H, m, PCH$_2$CH$_2$CH$_2$), 1.81-1.89 (3H, m, PCH$_2$CH$_2$CH$_2$ + CHH), 1.96-2.09 (4H, m), 2.15-2.23 (3H, m), 2.32-2.40 (1H, m), 2.45-2.56 (4H, m), 2.59-2.64 (3H, m), 2.70-2.76 (2H, m), 2.76-2.82 (2H, m), 2.87-2.97 (3H, m), 3.28-3.35 (2H, m), 3.36-3.38 (1H, m), 3.40-3.44 (1H, m), 3.46-3.54 (2H, m), 3.61-3.68 (1H, m), 3.72-3.77 (1H, m, H$^b$) 3.79-3.87 (2H, m, PCH$_2$CH$_2$CH$_2$), 3.99-4.07 (4H, m, P(OCH$_2$CH$_2$)$_2$), 4.13-4.19 (1H, m, H$^b$), 7.56 (1H, br, NH), 8.85 (1H, br, NH). $^{13}$C NMR (176 MHz, CDCl$_3$) $\delta$ 16.6 (d, $^3$J = 6, P(OCH$_2$CH$_2$)$_2$), 22.0 (d, $^2$J = 5, NCH$_2$CH$_2$CH$_2$P), 22.9 (d, $^1$J = 143, NCH$_2$CH$_2$CH$_2$P), 27.9, 28.0 (overlapping C(CH$_3$)$_3$),
35.3, 38.7, 44.5, 47.1, 50.2 (CHNH), 51.5 (d, \(J = 16\), NCH\(_2\)CH\(_2\)N), 52.8, 55.6, 55.9, 56.2, 60.7 (CH), 61.7 (overlapping d, \(J = 7\), P(OCH\(_2\)CH\(_2\)O)), 82.0, 82.1, 82.1, 82.3 (C(CH\(_3\))\(_3\)), 167.4, 167.8 (C=O), 172.6, 172.8, 172.9, 173.4, 175.3, 181.4, 181.8 (CO). \(^{31}\)P NMR (283 MHz, CDCl\(_3\)) \(\delta\) 30.85. MS (ES\(^+\)) \(m/z\) 1028.5 [M+H]\(^+\); C\(_{49}\)H\(_{87}\)N\(_7\)O\(_{14}\)P requires 1028.605; found 1028.606.

\([\text{Gd.L}^2]\)

![Conjugate 2](image)

The [Conjugate 2] (10 mg, 0.01 mmol) was dissolved in MeOH (200 \(\mu\)L) with stirring. To this was added NaOH (0.5 mg) as a solution in H\(_2\)O (2.5 M) and the resulting solution stirred at room temperature overnight. Complete removal of the methyl ester was verified by ESI-MS, at which point the solvent was removed under reduced pressure. The residue was completely dried under high vacuum before being suspended in DCM (1 mL), to which trifluoroacetic acid (1 mL) was added. The resulting yellow solution was stirred at room temperature overnight. Complete removal of the tert-butyl ester groups was verified by ESI-MS, at which point excess solvent was removed under reduced pressure. The residue was repeatedly re-dissolved in DCM (2 mL) and the solvent removed under reduced pressure to remove excess TFA. This process yielded the protonated salt of \(\text{L}^2\) as a pale-brown solid. MS (ES\(^+\)) \(m/z\) 698.3 [M+H]\(^+\). The salt of \(\text{L}^2\) (7.5 mg, 0.01 mmol) was dissolved in H\(_2\)O (0.5 mL) and the pH adjusted to about 5.5 by the addition of NaOH (0.1 M). GdCl\(_3\).6H\(_2\)O (4.8 mg, 0.013 mmol) was added as a solution in H\(_2\)O (0.5 mL) and the reaction mixture stirred at 60 °C overnight. The pH of the solution was periodically checked and maintained between 5-6 with the addition of NaOH/HCl (0.1 M). Upon completion of complexation, excess gadolinium was removed by the addition of Chelex-100™ with stirring. The Chelex trap was filtered and the complex eluted with excess H\(_2\)O. Removal of the water by lyophilisation gave a white solid that was purified by RP-HPLC. HR-MS (ES\(^+\)) C\(_{29}\)H\(_{41}\)\(^{154}\)GdN\(_7\)O\(_{13}\) requires 849.1971 [M+2H]\(^+\); found 849.1956. \(r_p = 4.54\) mM\(^{-1}\)s\(^{-1}\) (60 MHz, 310K). RP-HPLC: \(t_R = 7.1\) mins [2-30% MeOH in H\(_2\)O over 10 mins].

\([\text{Gd.L}^4]\)
[Conjugate 4] (17 mg, 0.02 mmol) was dissolved in DCM (1 mL) with stirring. To this solution was added trifluoroacetic acid (1 mL) and the resulting solution stirred at room temperature for overnight. Hydrolysis of the tert-butyl ester groups was verified by ESI-MS, at which point the excess solvent was removed under reduced pressure. The residue was repeatedly re-dissolved in DCM (2 mL) and the solvent removed under reduced pressure to remove excess TFA. This process yielded the phosphonate ethyl ester as a light-brown solid. This residue was dissolved in DMF (1 mL) to which bromotrimethylsilane (18 µL, 0.13 mmol) was added dropwise. The resulting mixture was heated to 60 °C overnight until complete hydrolysis occurred as indicated by ESI-MS. The solvent was removed under reduced pressure, before the residue re-dissolved in H₂O. The pH was adjusted to 6 and the aqueous phase washed with DCM (3 x 3 mL) and diethyl ether (3 x 3 mL). The aqueous solvent was then removed by lyophilisation to give the protonated salt of L₄ as a light brown solid. MS (ES⁺) m/z 748.7 [M+H]⁺. The salt of L₄ (12.5 mg, 0.017 mmol) was dissolved in H₂O (0.5 mL) and the pH adjusted to 5.5. GdCl₃.6H₂O (6.8 mg, 0.018 mmol) was added as a solution in H₂O (0.5 mL) and the reaction mixture stirred at 60 °C overnight. The pH of the solution was periodically checked and maintained between 5 and 6 with the addition of NaOH/HCl (0.1 M). Upon completion of complexation, excess gadolinium was removed by the addition of Chelex-100™ with stirring. The Chelex trap was filtered and the complex eluted with excess H₂O. Removal of the water by lyophilisation gave the complex, [Gd.L₄] as a white solid that was purified by RP-HPLC. HR-MS (ES⁺) C₂₉H₄₄₁⁵⁴GdN₇O₁₄P requires 899.1893 [M+2H]²⁺; found 899.1891. r₁p = 5.21 mM⁻¹s⁻¹ (60 MHz, 310K). RP-HPLC: tᵣ = 7.5 mins [2-30% MeOH in H₂O over 10 mins].

[Gd.L₄]
[Conjugate 6] (10 mg, 0.01 mmol) was dissolved in DCM (1 mL) with stirring. To this solution was added trifluoroacetic acid (1 mL) and the resulting solution stirred at room temperature for overnight. Hydrolysis of the tert-butyl ester groups was verified by ESI-MS, at which point the excess solvent was removed under reduced pressure. The residue was repeatedly re-dissolved in DCM (2 mL) and the solvent removed under reduced pressure to remove excess TFA. This process yielded the phosphonate ethyl ester as a light-brown solid. This residue was dissolved in DMF (1 mL) to which bromotrimethylsilane (10 µL, 0.07 mmol) was added dropwise. The resulting mixture was heated to 60 °C overnight until complete hydrolysis was indicated by ESI-MS. The solvent was removed under reduced pressure, before the residue re-dissolved in H₂O. The pH was adjusted to 6 and the aqueous phase washed with DCM (3 x 3 mL) and diethyl ether (3 x 3 mL). The aqueous solvent was then removed by lyophilisation to give the protonated salt of L¹² as a light brown solid. MS (ES⁺) m/z 847.8 [M+H]⁺. The salt of L¹² (7.6 mg, 0.01 mmol) was dissolved in H₂O (1 mL) and the pH adjusted to 6. GdCl₃·6H₂O (4.0 mg, 0.011 mmol) was added as a solution in H₂O (0.5 mL) and the reaction mixture stirred at 60 °C overnight. The pH of the solution was periodically checked and maintained between 5 and 6 with the addition of NaOH/HCl (0.1 M). Upon completion of complexation, excess gadolinium was removed by the addition of Chelex-100™ with stirring. The Chelex trap was filtered and the complex eluted with excess H₂O. Removal of the water by lyophilisation gave the complex as a white solid that was purified by RP-HPLC. HR-MS (ES⁺) C₃₄H₅₅¹⁵⁶GdN₆O₁₅P requires 1002.261 [M+2H]⁺; found 1002.267. r₁p = 5.18 mM⁻¹ s⁻¹ (60 MHz, 310K). RP-HPLC: t_R = 7.8 mins [2-30% MeOH in H₂O over 10 mins].

**Compound 26**
(2R)-1-tert-Butyl 5-methyl 2-(4,10-bis(2-(tert-butoxy)-2-oxoethyl)-7-((2R)-1-(tert-butoxy)-6-(4-((2-(diethoxyphosphoryl)ethyl)-8,9-dioxo,2,6-diazabicyclo[5.2.0]non-1(7)-en-4-yl)amino)-4-oxobutamido)-1-oxohexan-2-yl)-1,4,7,10-tetraazacyclododecan-1-yl)pentanediolate (50 mg, 0.042 mmol) was dissolved in methanol (1 mL) and NaOH (2 mg, 0.051 mmol) was added as a solution in H$_2$O (250 µL). The light yellow solution was stirred at room temperature and progress of the reaction monitored by ESI-MS. Upon complete hydrolysis of the methyl ester after 1 hour, the solvent was removed under reduced pressure to give a pale yellow solid, which was dried fully on a high vacuum line. This yielded the carboxylic acid (49 mg, 0.042), to which EDC (9.6 mg, 0.050 mmol) and HOBt (6.8 mg, 0.050 mmol) were added and dissolved in anhydrous DMF (1 mL). The mixture was stirred at room temperature under an atmosphere of argon for 20 minutes. After this period, a pre-stirred solution of $N$-(2-Aminoethyl)-5-(3aS, 4S, 6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide$^{16}$ (12 mg, 0.042 mmol) and NMM (9 µL, 0.083 mmol) in anhydrous DMF (0.5 mL) was added dropwise and the resulting solution stirred at room temperature until complete consumption of the starting materials was revealed by ESI-MS. After this period, the solvent was removed under reduced pressure and the crude oil taken up into EtOAc (15 mL). NaHCO$_3$ (15 mL) was added, the layers separated and the aqueous washed with EtOAc (3 x 20 mL). The combined organic portions were dried over MgSO$_4$, filtered and the solvent removed under reduced pressure. The crude residue was purified by column chromatography (DCM/MeOH/NH$_4$OH, 100% to 80:15:5; $R_f$=0.15) to yield a yellow oil (10 mg, 17%). $^1$H NMR (700 MHz, CDCl$_3$) δ 1.29-1.37 (6H, m, P(OCH$_2$CH$_3$)$_2$), 1.40-1.51 (40H, m), 1.56-1.75 (5H, m), 2.22-2.80 (20H, m), 2.80-3.51 (31H, m), 3.60-3.93 (5H, m), 5.05-4.15 (4H, m, P(OCH$_2$CH$_3$)$_2$), 4.21-4.52 (3H, m), 7.87-8.51 (3H, m). $^{31}$P NMR (283 MHz, CDCl$_3$) δ 27.17. MS (ES$^+$) $m/z$ 727.4 [M+2H]$^{2+}$; $C_{68}H_{118}N_{12}O_{18}$PS requires 1453.815; found 1453.819.

[Gd.L$^7$]

Compound 26 (10 mg, 0.007 mmol) was dissolved in DCM (1 mL) with stirring. To this solution was added trifluoroacetic acid (1 mL) and the resulting solution stirred at room temperature for overnight. Hydrolysis of the tert-butyl ester groups was verified by ESI-MS.
at which point the excess solvent was removed under reduced pressure. The residue was repeatedly re-dissolved in DCM (2 mL) and the solvent removed under reduced pressure to remove excess TFA. This process yielded the phosphonate ethyl ester as a light-brown solid. This residue was dissolved in DMF (0.8 mL) to which bromotrimethylsilane (7 µL, 0.055 mmol) was added dropwise. The resulting mixture was heated to 60 °C overnight until complete hydrolysis as verified by ESI-MS. The solvent was removed under reduced pressure, before the residue re-dissolved in H2O. The pH was adjusted to 6 and the aqueous phase washed with DCM (3 x 2 mL) and diethyl ether (3 x 2 mL). The aqueous solvent was then removed by lyophilisation to give the protonated salt of L7 as a light brown solid. MS (ES') m/z 587.0 [M+2H]2+. The salt of L7 (8.1 mg, 0.007 mmol) was dissolved in H2O (0.5 mL) and the pH adjusted to 6. GdCl3.6H2O (3.1 mg, 0.008 mmol) was added as a solution in H2O (0.5 mL) and the reaction mixture stirred at 60 °C overnight. The pH of the solution was periodically checked and maintained between 5 and 6 with the addition of NaOH/HCl (0.1 M). Upon completion of complexation, excess gadolinium was removed by the addition of Chelex-100™ with stirring. The Chelex trap was filtered and the complex eluted with excess H2O. Removal of the water by lyophilisation gave the complex as a white solid that was purified by RP-HPLC. HR-MS (ES') C48H75154GdN12O18PS requires 1324.399 [M+2H]+; found 1324.399. r1p = 7.23 mM⁻¹ s⁻¹ (60 MHz, 310K). RP-HPLC: tR = 16.7 mins [2-30% MeOH in H2O over 10 mins].

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Notes and references


