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Self-Immulative Activation of β -galactosidase Responsive Probes for In Vivo MR Imaging in Mouse Models

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Abstract

Our lab has developed a new series of self-immulative MR agents for the rapid detection of enzyme activity in mouse models expressing β -galactosidase (β -gal). We investigated two molecular architectures to create agents that detect β -gal activity by modulating the coordination of water to Gd(III). The first is an intermolecular approach, wherein we designed several structural isomers to maximize coordination of endogenous carbonate ions. The second involves an intramolecular mechanism for q modulation. We incorporated a pendant coordinating carboxylate ligand with a 2, 4, 6, or 8 carbon linker to saturate ligand coordination to the Gd(III) ion. This renders the agent ineffective. We show that one agent in particular (6-C pendant carboxylate) is an extremely effective MR reporter for the detection of enzyme activity in a mouse model expressing β -gal.

Graphical Abstract

In vivo detection of β -gal activity in mammals. The agent was injected intraperitoneally into transgenic LacZ mice and global signal enhancement was observed 1 hr post injection. At 24h, the MR signal returned to baseline suggesting that the agent is cleared renally. No signal enhancement is observed in non-transgenic native control mice.

Keywords

β -galactosidase; Imaging agents; Macrocyclic Ligands; Magnetic Resonance; Self Immolative

Introduction

Magnetic resonance imaging (MRI) is a powerful clinical and research modality capable of excellent spatiotemporal resolution and an unlimited depth penetration.^[1] It is the modality of choice for evaluating many diseases, and is a staple of clinical diagnostic radiology due to its tunable soft-tissue contrast, high spatial and temporal resolution, and lack of ionizing radiation.^[2] An observed signal is derived from water protons and surrounding tissue; this technique provides high quality tomographic images with an impressive resolution.^[3]

In order to differentiate regions that are magnetically similar but histologically distinct, paramagnetic probes or contrast agents (CAs) are commonly used. CAs have proven indispensable for the diagnosis and monitoring of disease states.^[4] Further, they have become an invaluable research tool for developing our understanding of fundamental biological processes and in the investigation of pathophysiology.^[4d]

Chelated Gd(III) is the most commonly used for MR CAs.^[4a, 4b, 5] Gd(III) shortens the intrinsic T_1 relaxation rates of water, which results in positive contrast in an MR image. T_1 modulation is largely due to Gd(III) possessing a slow electron relaxation time and $S = 7/2$ ground state. While free Gd(III) is toxic, it can be made biocompatible by using strong chelating agents with $K_{DS} \sim 10^{25.3} \text{ M}^{-1}$ for $[\text{Gd(III)DOTA}]^-$ measured at ambient temperature.^[6] The efficiency of Gd(III) chelates at shortening the T_1 of H_2O depends on the chelates physiochemical properties, and is referred to as relaxivity ($r_1 \text{ mM}^{-1}\text{s}^{-1}$). The observed signal intensity of an MR contrast agent can be modulated by varying one of several physical parameters including: q , the number of bound waters to Gd(III), τ_r , the rate of molecular tumbling, and τ_m , the rate of water exchange.^[4b]

During the last two decades, MR CAs have been transformed from purely anatomical reporters into bioactivated or bioresponsive probes.^[7] These probes are designed to be conditionally activated, and therefore provide information not available from traditional MR CA's. The impetus behind the development of these agents is to enable the *in vivo* monitoring of enzyme activity, gene expression, endogenous metal ions, pH and redox events.^[8] There are several reported mechanisms for how bioresponsive agents function. We have focused on the modulation of the inner-coordination sphere water molecules, or q . This class of chelating ligands is designed to have two distinct states with respect to inner-sphere water coordination: $r_{1,\text{off}}$, where the Gd(III) is coordinatively saturated and the resulting image appears "darker", and $r_{1,\text{on}}$, where water coordinates to an open site on a Gd(III) ion

resulting in a brighter image. Relaxation enhancement by a Gd(III) chelate is described by Eq. 1, with a larger r_1 corresponding to a more effective or sensitive contrast agent:

$$\frac{1}{T_{1, \text{obs}}} = \frac{1}{T_{1, \text{d}}} + r_1[\text{Gd}^{\text{III}}] \quad \text{Eq. 1}$$

One of the primary goals of molecular imaging is to visualize molecular events in whole animals.^[4d] This has required the development of bioresponsive probes to monitor gene expression, and is vital for the understanding of pathophysiology in numerous diseases, including cancer.^[9] A particularly successful approach for monitoring *in vivo* gene expression is to insert a marker gene to record regulation and expression. Gene reporters have played a significant role in molecular biology and biomedical research, and are traditionally used in combination with histological techniques.^[10] The bacterial *lacZ* gene produces the enzyme β -galactosidase (β -gal) and has been historically important as a gene reporter.^[11] Typically, *in vivo* detection of *lacZ* has been accomplished using colorimetric assays that require the organism be sacrificed and the tissues of interest be stained for the presence of β -gal. To overcome this limitation, we designed a series of β -gal activated MR contrast agents employing a *q* modulation mechanism that successfully detected gene expression *in vitro* and in *Xenopus laevis*.^[7a, 12] Since then, a number of excellent examples of bioresponsive agents have emerged for MR imaging detection of β -gal activity.^[13]

Here we describe a platform architecture based on our earlier work with β -glucuronidase, and we have optimized the chemistry to allow for the substitution of the pendant sugars.^[14] We have investigated two mechanisms for *q*-modulated contrast agents activated by β -gal (Figure 1). The first is an intermolecular approach, wherein we designed a series of structural isomers to attempt to maximize coordination of endogenous CO_3^{2-} . The second approach involves an intramolecular mechanism in which we incorporated a pendant coordinating carboxylate ligand with various linker lengths to optimize coordination within the same chelate scaffold. Both of these designs employ a self-immolative linker that undergoes an electron cascade when the galactose moiety is hydrolyzed by β -gal.^[15] This carbamate linker was selected for its rapid kinetics; the galactose substrate is positioned away from the sterically bulky Gd(III) chelate, allowing for easier access by the enzyme.^[15] Byproducts of activation include CO_2 gas, galactose, and either a nitrophenol (intermolecular) or an alkylated aniline (intramolecular).

The intramolecular activation strategy employed the *para*-configuration of the self-immolative linker to maximize activation kinetics.^[16] A pendant coordinating carboxylate was incorporated *via* the aniline (reduced from the nitro group) with a 2, 4, 6, or 8 carbon linker (2C, 4C, 6C, 8C respectively). The challenge in achieving intramolecular coordination is to impart enough flexibility to the linker to create a situation where the carboxylate coordination is more favorable than either carbamate or intermolecular CO_3^{2-} coordination. In the presence of the enzyme, both the inter- and intramolecular mechanisms self-immolate to the identical product (**11**), which was independently synthesized and characterized in this study.

MATERIALS AND METHODS

General Considerations

HPLC-grade acetonitrile, methanol, hexanes, ethyl acetate, dichloromethane, and 70% nitric acid were obtained from VWR. Cyclen, $\text{GdCl}_3 \cdot 6\text{H}_2\text{O}$, and Pd/C (10 wt. %) were obtained from Strem. Ethyl 8-bromooctanoate was obtained from Santa Cruz Biotechnology. Hydrogen was obtained from Airgas. Triethylamine, N,N-diisopropylethylamine, and acetonitrile were dried using a Glass Contour solvent system. Water was obtained from a Millipore Milli-Q Synthesis purifier. All other reagents and solvents were purchased from Sigma Aldrich and used without further purification. Standard grade 60 Å, 230–400 mesh silica gel (Sorbent Technologies) was used for flash chromatography. Thin layer chromatography (TLC) was performed on EMD 60F 254 silica plates (Machery Nagel). Compounds containing unmetalated cyclen derivatives were visualized with an iodoplatinate stain, while all other compounds were visualized with Hanessian's Stain and ninhydrin. NMR spectra were collected on a Bruker Avance III 500 MHz spectrometer equipped with DHC CryoProbe, all spectra are referenced to CDCl_3 . Electrospray ionization mass spectrometry (ESI-MS) was performed on a Bruker AmaZon X equipped with an Agilent 1200 series HPLC system and a quadrupole ion trap and high-resolution MS spectra were collected on an Agilent 6210A LC-TOF. The Gd(III) analogues of the cleaved agent (**11**) were synthesized as previously reported. The Tb^{III} analogue of this was synthesized in the same manner but metalated with TbCl_3 . All complexes were purified by semi-preparative HPLC. The synthesis of ethyl DO3A-HBr and TLC stain preparation can be found in Supporting Information, page S2.

Synthesis

Details of the synthesis and characterization of all the intermediates and complexes described in this study can be found in Supporting Information.

HPLC Purification

All metalated complexes were purified by semi-preparative HPLC on a Varian Prostar 500 system equipped with a Varian 335 UV-vis and an HP 1046 fluorescence detector. Compound purification was verified by analytical HPLC on a Varian Prostar 500 system equipped with a Varian Prostar 335 UV-vis and Varian Prostar 363 fluorescence detector. Both systems utilized a binary mobile phase of MilliQ water (A) and HPLC-grade MeCN (B). All samples were injected in 100% water and elution gradients were run from 5 to 50% MeCN. Compounds **5a-c** and **5a'-c'** were purified/verified using a Waters $19 \times 250 \text{ mM } 5 \mu\text{M}$ Atlantis T3 C18 column or Waters $4.6 \times 250 \text{ mM } 5 \mu\text{M}$ Atlantis T3 C18 column, for semi-preparative and analytical respectively. Compounds **10a-d** and **10a'-d'** were purified/verified using a Phenomenex Synergi $4 \mu\text{M}$ Polar-RP 80Å LC Column $150 \times 21.2 \text{ mm}$.

Inductively Coupled Plasma – Mass Spectrometry (ICP-MS)

Precise Gd(III) concentrations used for relaxivity and MR imaging were determined using ICP-MS. Samples were prepared by digestion in concentrated HNO_3 (100 μL) at 80 °C in 15 mL conical tubes for 1 hour followed by dilution to a total volume of 10 mL with MilliQ

water. Samples were analyzed on a computer-controlled (Plasmalab software) Thermo X series II ICP-MS (Thermo Fisher Scientific, Waltham, MA) equipped with an ESI SC-2 autosampler (Omaha, NE). Each sample was acquired using one survey run (10 sweeps) followed by three main (peak jumping) runs (100 sweeps). Isotopes analyzed included $^{154,157,158}\text{Gd(III)}$, while ^{115}In and ^{165}Ho were used as internal standards for data interpolation and instrument stability.

Relaxivity (1.4 T)

T_1 relaxation times were obtained for **5a-c** (Figure S5), and complexes **10a-d** (Figure 3), and **11** at 37 °C at 60 MHz (1.4 T) on a Bruker mq60 minispec spectrometer (Billerica, MA). Three 1 mM solutions of each complex were prepared in four different buffer conditions 1) 10 mM 3-(N-morpholino)propanesulfonic acid (MOPS), 100 mM NaCl, pH 7.4, 2) 10 mM MOPS, 24 mM NaHCO_3 , 100 mM NaCl, pH 7.4, (3) Dulbecco's Phosphate Buffer (DPBS) pH = 7.3 Life Technologies, and 4) β -galactosidase enzyme assay buffer, 200 mM sodium phosphate, 100 mM β -mercaptoethanol, 2 mM MgCl_2 , pH = 7.3 (Figure S6). Three sets of five serial dilutions (~ 1 mM, 0.5 mM, 0.25 mM, 0.125 mM and 0.0625 mM) were prepared for each complex. T_1 relaxation rates were measured using an inversion recovery pulse sequence (t1_ir_mb) with a 10 ms first pulse separation, recycle delay and final pulse separation $\geq 5 T_1$. T_1 relaxation rates were plotted as a function of Gd(III) concentrations as determined by ICP-MS, r_1/r_2 was obtained from linear fit of concentration vs. T_1 as described in Eq. 1.

Complex stability was assessed by relaxivity (T_1) for **10a-d** by measuring 1 mM solutions in enzyme buffer (200 mM Na_3PO_4 , 100 mM β -mercaptoethanol, 2 mM MgCl_2 , pH 7.3) and compound **10c** was additionally measured in cell culture media over the course of two weeks (Figure S7). No significant change in T_1 was observed over 140 hours.

Terbium(III) Luminescence: determination of q .

The number of water molecules directly coordinated to the lanthanide center (q), was determined for each of the Tb^{III} analogues **5a'-c'** (See Table S2), **10a'-d'** (Table 1), and **11'** in MilliQ water and MOPS/carbonate buffer (10 mM MOPS, 24 mM NaHCO_3 , pH 7.4). The monoexponential fluorescence decay of an ~ 1 mM solution of each complex was determined using a Hitachi F4500 fluorometer (Schaumburg, IL). An excitation wavelength of 395 nm and an emission wavelength of 614 nm were used, and 25 scans were acquired and averaged for each solution. The averaged data was fit with a monoexponential decay function, and the time constant (τ) for each solution was determined in triplicate. The fluorescence decay curves of the complexes were measured in both H_2O and D_2O , and in both buffer and deuterated buffer - obtained by lyophilizing and re-suspending the buffer in D_2O three times (Figure S8). The q values were calculated using the empirically derived Horrock's equation for Tb^{III} (Eq. 2):^[17]

$$q = 4.2 \left(\frac{1}{\tau_H} - \frac{1}{\tau_D} - 0.06 \right) \quad \text{Eq. 2}$$

Enzyme Kinetics (UV-vis)

Enzyme activation kinetics were determined by the method of initial rates for compounds **5a-c** via the appearance of the nitrophenol byproduct of activation. The characteristic absorption was monitored at 420 nm. Concentrations of substrate used were 0, 0.05, 0.1, 0.12, 0.15, 0.3, 0.4, 0.5, 0.75, 1, and 2 mM. Each concentration was measured in triplicate and exact concentrations were measured by ICP-MS (See Table S1)

MR Imaging Solution Phantoms (+/- β -gal) (7 T).

MR images were acquired on a Bruker Pharmscan 7 T imaging spectrometer fitted with shielded gradient coils at 25 °C. Image analysis was carried out using Paravision 5.0 pl3 software (Bruker, Billerica, MA, USA) with monoexponential curve-fitting of image intensities of selected regions of interest (ROIs) for each axial slice.

Complexes **10b**, **10c**, **10d**, and **11** were prepared in enzyme assay buffer (200 mM sodium phosphate, 100 mM β -mercaptoethanol, 2 mM MgCl_2 , pH 7.3) at 500 μM . Exact Gd(III) concentration was verified by ICP-MS. Either 10 μL of enzyme stock solution (50% enzyme assay buffer, 50% glycerol, at 521 U/mL β -gal) or 10 μL of a 50% enzyme assay buffer/50% glycerol solution (used as a control) were added to a 190 μL aliquot of each complex. After this addition, the final concentration of each complex was 475 μM , with 26.1 U/mL of β -gal. The solutions were incubated at 37 °C for 24 hours, then aliquoted to 5, $\frac{3}{4}$ " flame-sealed Pasteur pipets. The bottom sections of the flame-sealed pipets were then scored with a glass scribe, broken into small capillaries, and imaged using a RF RES 300 1H 089/023 quadrature transmit receive 23 mm volume coil (Bruker BioSpin, Billerica, MA, USA). T_1 relaxation times were measured using a rapid-acquisition rapid-echo RARE-VTR T_1 -map pulse sequence with the following imaging parameters: TE (11.0 ms), TR (500.0 ms), field of view (FOV) = $25 \times 25 \text{ mm}^2$, matrix size (MTX) = 256×256 , number of axial slices = 5, slice thickness (SI) = 1.0 mm, and averages (NEX) = 3, and total scan time was 2:36:57. T_2 values were measured using a multislice multiecho (MSME) T_2 -map pulse sequence, with static TR (5000 ms) and 32 fitted echoes in 11 ms intervals (11, 22, 352 ms). Imaging parameters were as follows: FOV = $25 \times 25 \text{ mm}^2$, MTX = 256×256 , number of axial slices = 4, SI = 1.0 mm, NEX = 3, and a total scan time = 48 min. T_1 images and times can be found in Figure 3 and T_2 maps and times can be found in Figure S9.

In vivo MR Imaging (9.4 T)

Hemizygous transgenic mice constitutively expressing the *lacZ* gene that encodes the β -gal enzyme (strain ROSA26-*lacZ*) were purchased from The Jackson Laboratory (Bar Harbor, ME). A non-transgenic littermate was used as a control. 200 μL of 40 mM **10c** was injected into 3 mice in the intraperitoneal cavity (IP). Mice were imaged with MRI at baseline, and 1, 4, and 24 hours post injection.

MRI was performed on a 9.4T Bruker Biospec MRI system with a 30 cm bore, a 12 cm gradient insert, and an Autopac automated sample positioning system (Bruker Biospin Inc, Billerica, MA). Animals were anesthetized in an induction chamber with 3% isoflurane and transferred to a dedicated imaging bed with isoflurane delivered via nosecone at 1–2%. Respiratory signals were monitored using an MR-compatible physiologic monitoring system

(SA Instruments, Stonybrook, NY) and a warm water circulating system was used to maintain body temperature. Animals were placed in the prone position and a quadrature radiofrequency coil with inner diameter 40mm was mounted on the bed. Mice were imaged using a FLASH gradient echo sequence using the following parameters: TR/TE/ α = 100 ms / 2.2 ms / 45°, MTX = 192 × 192, FOV 4 × 4 cm, 9 slices of 1 mm thick, 0.3mm slice gap, and 1 signal average.

RESULTS AND DISCUSSION

The fundamental aim of this work is the development of a platform architecture for bioresponsive MR agents that can be activated by several enzymes and in this particular case we focused on β -gal. We synthesized and characterized two different architectures of the β -gal activated contrast agent (Figure 1). In the *intermolecular* design, a carbonate is occupying the open coordination site of the Gd(III) ion (Figure S1). In the *intramolecular* design the ninth coordination site is occupied by a pendent carboxyl ligand attached to the *para* position of the nitrobenzene (Figure 2).

Based upon our results with the intermolecular design, we selected the *para* sugar configuration due to the rapid enzyme kinetics and the relative synthetic ease of incorporating a pendant carboxylate ligand (Figure 2 and Figure S3). In this strategy, the nitro group was reduced to the corresponding aniline for synthetic incorporation of a pendant carboxylate arm with variable levels of flexibility. Aliphatic linkers composed of 1,3, 5, or 7 carbons (**10a-d**) were used to connect the pendant carboxylate to the aniline nitrogen in an effort to determine the optimal flexibility for facilitating intramolecular coordination to the Gd(III) ion. Although this approach is known to nominally decrease the catalytic rate due to the electron induction into the ring, we were confident this decrease would result in facile kinetics and an increase in the change in r_1 .^[18]

q measurements (intramolecular ligand coordination)

As with the intermolecular series, the Tb^{III} luminescence properties of **10a'-d'** were used to determine q in water *versus* a MOPS/CO₃²⁻ buffer (Table 1). If intramolecular coordination is more favorable than the intermolecular coordination, the inner-coordination sphere should not change in the presence of the strongly coordinating CO₃²⁻ anion. These data suggest that the 2C and 4C linkers (**10a'**, **b'**) were too short and inflexible to coordinate to the lanthanide center, making them unsuitable for q -modulation. However, both the 6C and 8C linkers (**10c'** and **10d'**), respectively, have a $q = 0.1$ in the presence of CO₃²⁻. This result suggests that in these complexes there is sufficient flexibility in the linker to facilitate coordination of the pendant carboxylate, making these the best candidates for intramolecular activation and maximization of r_1 .

Relaxivity (intramolecular design):

Relaxivities of the intramolecular series were measured at 1.4 T to evaluate signal changes in the absence and presence of CO₃²⁻. Measurements were conducted in two buffer conditions, MOPS and MOPS/CO₃²⁻ (Figure 3a), and plotted as percent change in signal r_1 (Figure 3b). As expected based on the measured q values, **10a** has the highest relaxivity

of the series, suggesting that intramolecular coordination between the pendant carboxylate and the Gd^{III} ion is not significant. As a result, **10a** displays a negative r_1 . **10b** displays the largest r_1 in MOPS and **10c** demonstrates the highest overall change in signal in the presence of CO_3^{2-} . Although the 8C linker in **10d** can coordinate the lanthanide ion ($\text{Gd}(\text{III})$ or $\text{Tb}(\text{III})$), this agent displays a negative r_1 and is therefore not viable for *in vivo* imaging.

Solution Phantom MR Imaging, +/- β -gal:

Based on the results from the r_1 and q measurements, we selected **10b**, **10c**, and **10d** for further investigation by MR imaging at 7 T. **10b** was selected because it displayed a positive r_1 , even though it did not display intramolecular q -modulation. **10c** was selected as the optimal candidate, displaying both a positive (20%) r_1 and effective q -modulation. **10d** was selected because it displayed effective q -modulation even though the r_1 was negative at 1.4 T.

T_1 solution phantom images were collected in the presence and absence of β -gal (Figure 4). **10b** displayed the greatest change in MR contrast but this does not arise from intramolecular q -modulation, as determined by Tb^{III} luminescence where the $q = 0$ in pure water (Table 1). However, **10b** does not appear to fully activate in the presence of β -gal, as the $T_1 + \beta$ -gal (378 ms) did not reach the T_1 of activated agent (**11**: 334 ms). **10c** and **10d** have approximately the same T_1 signal in the presence of β -gal as **11** (See Figure 1).

MR Imaging in Transgenic Mice:

Based on these results, we selected complex **10c** (6C pendant carboxylate ligand) for *in vivo* studies in mice. This complex has a $q = 0.1$ in the presence of CO_3^{2-} and a positive change in relaxivity at both 1.4 and 7 T. **10c** was injected intraperitoneally into transgenic LacZ mice (Figure 5: **Rows 1 and 2**: Jackson Labs: <http://www.jax.org>). Global signal enhancement was observed in the lacZ-expressing mice at 1 hr post injection (prominent in the liver and kidney. At 4 hrs post injection, signal enhancement was present but at a reduced level. In one LacZ mouse, the bladder was visible at the 4 hr time point with a very bright MR signal, suggesting that the β -gal responsive MR agent is cleared renally. In both mice, the MR signal returned to baseline within 24 hours, suggesting that the bulk of the agent was cleared by the kidney. No signal enhancement was observed in non-transgenic native control mice (Figure 5: **row 3**).

CONCLUSIONS

In this report, we have synthesized, characterized and evaluated two structural designs for the preparation of q -modulated, self-immolative activated MR contrast agents for the *in vivo* detection of β -galactosidase. The first exploits intermolecular CO_3^{2-} to coordinatively saturate the $\text{Gd}(\text{III})$ ion to create an ineffective agent. The results from this investigation guided the strategy of a second design that involves the intramolecular coordination of a pendant carboxylate ligand with varied linker lengths. Both strategies exploit the rapid enzyme kinetics of a self-immolative linker that undergoes an electron cascade upon hydrolysis of the galactose moiety by β -gal.

For the intermolecular approach, we systematically interrogated a number of structural isomers designed to increase CO_3^{2-} affinity to the Gd(III) ion. This was accomplished by increasing steric interactions between galactose and the macrocycle. Even so, this intermolecular approach did not provide significant changes in relaxivity for the *in vivo* detection of β -gal activity. However, the significant improvement in the enzyme kinetics of **5c** (*para*), and the positive change in q , were important parameters to guide our intramolecular design. By inserting a pendent carboxylic functional group as part of the self-immolative linker, we were able to create a stable carboxylate coordination to the Gd(III) ion.

We have demonstrated that **10c** (6-C pendant carboxylate) is an extremely effective MR reporter for the detection of enzyme activity in a mouse model expressing β -gal. Based on the *in vivo* success of this approach, we are collaborating with a team of researchers for detecting gene therapy treatment in real-time. The need for non-invasive, disease specific biomarkers that reflect treatment efficacy is paramount in this field. Therefore, we are currently developing bioactivated MR agents using our intramolecular approach described here for Adeno-Associated Virus (AAV) treatment in Tay-Sachs/Sandhoff (GM2 gangliosidoses), hexosaminidase (or Hex-A), Pompe disease (α -glucosidase) and mucopolysaccharidosis VII (β -glucosidase).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1:

Inter- versus intramolecular activation of q -modulated MRI contrast agents sensitive to β -gal. The *intermolecular* design maximizes CO_3^{2-} binding by manipulating the structural isomers. The *intramolecular* design incorporates a pendant carboxylate ligand where the linker length was varied to maximize coordination. Both designs feature a self-immolative linker that improves activation kinetics. An electron cascade (red arrows) for both designs are initiated by sugar hydrolysis. The intermolecular complex data and results are described in Supporting Information.

Figure 2:
Synthesis of the intramolecularly coordinated Gd(III) CAs activated by β -gal. Final metalated complexes (TbCl₃ or GdCl₃) were obtained *via* purification by preparative HPLC (see Supporting Information pages S6–S12).

Figure 3:

a) Relaxivity values of **10a** - 2C linker (black), **10b** - 4C linker (green), **10c** - 6C linker (blue), **10d** - 8C linker (red) and **11** - cleaved (white) in either MOPS or $\text{MOPS}/\text{CO}_3^{2-}$ at 37 °C and 1.4 T. **b)** r_1 values for **10a-d** relative to the cleaved control product **11** (see Figure 1).

Figure 4:

MR solution phantoms of **10b**, **10c**, **10d**, and **11** +/- enzyme at 7 T. A representative slice is shown and the T_1 values are calculated from the average of 5 independent slices. Standard deviations are reported in parenthesis. Images were acquired at ambient temperature. + β -gal samples were incubated with 26.1 U/mL of enzyme in assay buffer for 24 hours.

Figure 5:

Transgenic mice constitutively expressing LacZ (rows 1 and 2: Jackson Labs), and control mice (row 3) were injected intra-peritoneally (IP 200 microliters of 40 mM beta-gal agent ~8mmols – not sure of MW) with a β -galactosidase responsive agent where $n = 5$. Coronal MR images (9.4T) were acquired at preinjection, and 1, 4, and 24 hours post injection using a T_1 weighted FLASH sequence. Scan parameters: TR/TE/alpha = 100 ms/2.2 ms/45 deg, 192×192 matrix, 0.234 mm in-plane image resolution, and 1 mm slice thickness. Signal enhancement was noted in the liver and kidneys of the LacZ-expressing mice at 1 hr post injection, returning to baseline by 24 hrs. This signal enhancement was minimal for the control mice (row 3) except for mild enhancement in the kidneys post injections.

Table 1:

Luminescent decay properties and q values of Tb^{III} analogues **10a'**, **10b'**, **10c'**, and **10d'**. Decay (τ) rates were measured in H₂O, D₂O, MOPS/CO₃²⁻ buffer (τ_{Hbuf}), and deuterated MOPS/CO₃²⁻ buffer at pH = 7.4 (τ_{Dbuf}). The excitation wavelengths that were used are reported. q values were calculated using Horrock's equation for Tb^{III}. Emission was observed at 543 nm at ambient temperature.

Complex	Ex. λ (nm)	$\tau_{\text{H}_2\text{O}}$ (ms)	$\tau_{\text{D}_2\text{O}}$ (ms)	τ_{Hbuf} (ms)	τ_{Dbuf} (ms)	q (H ₂ O)	q (buffer)
10a'	308	1.1(4)	2.1(0)	1.7(2)	2.1(8)	1.3	0.3
10b'	308	1.6(3)	2.2(6)	1.8(9)	1.8(8)	0.5	0
10c'	310	1.8(9)	2.2(9)	1.9(3)	2.2(6)	0.1	0.1
10d'	310	1.9(3)	2.2(6)	1.9(7)	2.0(2)	0.1	0.1