

Supplementary Information for:

A second generation MRI contrast agent for imaging zinc ions *in vivo*

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1. General experimental conditions

Dansylsarcosin was provided by Eul Hyun Suh. Warfarin and male human blood serum were purchased from Sigma and FluoZinTM-1 tripotassium salt was obtained from Invitrogen, Carlsbad, CA. All other reagents and solvents were acquired from commercial sources and were used as received.

¹H- and ¹³C-NMR spectra were recorded on a Varian Gemini: Unity plus 200 MHz. Data are reported in the following order: chemical shift in ppm (δ); multiplicities are indicated as b (broadened), s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet); coupling constants, J, are reported in Hz; integration is provided.

Mass spectra (MS) were measured with a Voyager-DE PRO Biospectrometry Workstation (Applied Biosystems) [matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF)] operating in reflector mode using α-cyano-4-hydroxycinnamic acid as the matrix.

Analytical and semi-preparative HPLC was performed on a Hewlett Packard Series 1200 system using a Jupiter 5μ C18 300 Å 4.6 mm x 250 mm 5 microns (for analytical) or Jupiter 10μ C18 300 Å 10 mm x 250 mm 5 microns column (for semi-preparative) from Phenomenex. Typical separation procedure was carried out at room temperature with an isocratic or gradient of water/acetonitrile containing 0.1%/0.08% Trifluoroacetic acid (TFA) respectively unless indicated otherwise. Detection was done at 215 and 250 nm and a flow of 1 mL min⁻¹ (analytical) or 4.7 mL min⁻¹ (semi-preparative) was used.

T₁'s were measured using a Maran Ultra NMR relaxometer operating at 23 MHz (Oxford Instruments Molecular Biotools, Austin, TX). Longitudinal relaxivity values were determined from the slope of the line of the reciprocal of T₁ versus the concentration of gadolinium. Longitudinal relaxivity values were determined from the slope of the line of the reciprocal of T₁ versus the concentration of gadolinium according to equation (1)

$$r_1 = \frac{1}{T_1} [GdDOTA diBPYREN] + \frac{1}{T_0} \quad (1)$$

where T₀ is the diamagnetic relaxation time which was set constant to 3.2 s as determined at 23 MHz for a solution of Tris buffer 0.1 M, pH 7.6 at 37 °C.

A 5 mM solution of the gadolinium complex was made up in Tris buffer 0.1 M pH 7.6. This was serially diluted four times to give five different sample concentrations (5, 4, 3, 2, and 1 mM) at a [Gd]:[Zn] ratio of 1:0. Solutions containing gadolinium complex plus ZnCl₂ (CuCl₂) were also prepared to give a [Gd]:[Zn] ratio of 1:0.5. This titration was repeated until a 1:3 ([Gd]:[Zn]) ratio was reached. After 30 min of incubation at 37°C, T₁ measurements were made. Similar titration experiments were done with CaCl₂ and MgCl₂ to test the effects of competing cations. The same experiments were repeated using buffer containing HSA 600 μM but in this case the concentration of the gadolinium complex was varied from 1.2, 1, 0.8, 0.6 and 0.4 mM. For solutions containing HSA a high concentration of M²⁺, where M = Zn, Cu, Ca or Mg (>3.6 mM, that is for a [Gd]:[M] ratio of 1:3) causes protein precipitation. T₁'s measurements in male human blood serum (HBS) were done by reconstituting the lyophilized HBS in

MilliQ grade water first. The Gd complex was dissolved in the HBS solution and then it was serially diluted to give five different sample concentrations (5, 4, 3, 2, and 1 mM) at a [Gd]:[Zn] ratio of 1:0. Solutions containing gadolinium complex plus ZnCl₂ in HBS were also prepared to give a [Gd]:[Zn] ratio of 1:2.

All mouse treatments were done according to the standards of the UT Southwestern Institutional Animal Care and Use Committee (IACUC). Male blk6 normal mice at 8-10 weeks old were obtained for use in this study and conditioned in their new environment for at least 2 weeks in the regular chow diet before any feeding regimen and imaging were done.

All MR images (fast spin-echo multi-slice sequence, FSEMS) were obtained on a 9.4 T (400-MHz) horizontal-bore Varian INOVA imaging system with a dual-channel 38-mm diameter birdcage volume coil.

ICP-OES Gd³⁺ analysis were done by Galbraith Laboratories, Inc (Knoxville, TN).

All fluorescence measurements were performed in SpectraMax M5 Microplate Reader from Molecular Devices.

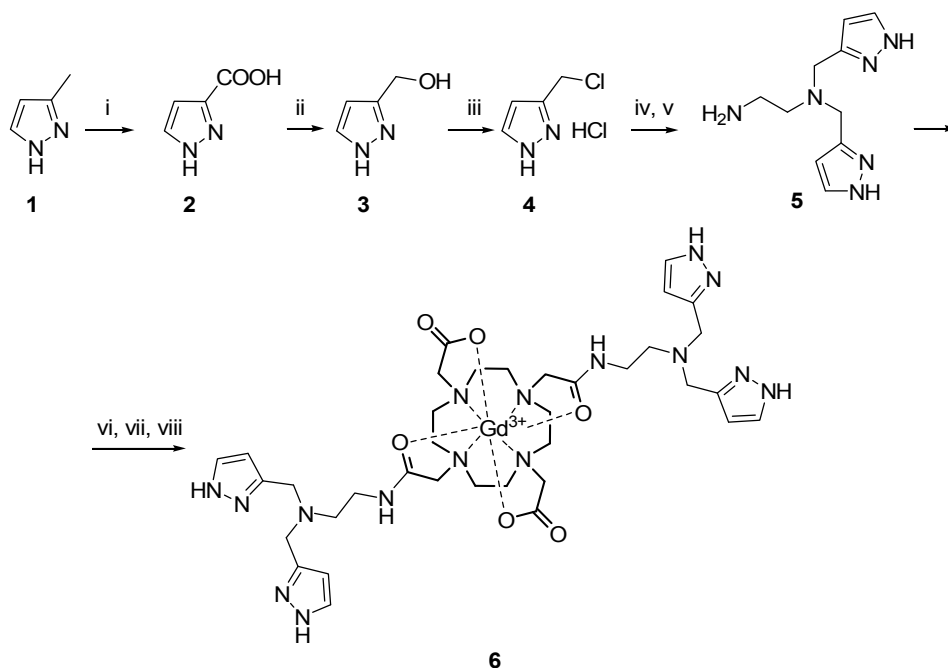
Curve fitting was done with GraphPad Prism Software Version 5.04 (Graph Pad Software Inc.).

Image analyses were carried out using ImageJ 1.41o software provided by the National Institutes of Health, USA.

2. Synthesis

The synthesis of 1,4,7,10-Tetraazacyclododecane-1,4 -bis(*tert*-butyl acetate)-7,10-acetic acid (DOTA-bis-*tert* butyl ester), was accomplished according to *De León-Rodríguez et al.*¹ The characterization data (¹H and ¹³C NMR, MS-ESI, elemental analysis) were in agreement with the proposed structure and what is reported.

The synthesis of 1,4,7,10-Tetraazacyclododecane-1,4 -bis(N,N-bis-(3-pyrazolyl-methyl) aminoethyleneacetamide)-7,10-acetic acid, (DOTAdiBPYREN) was accomplished following the procedure described below:



Scheme S1. i) KMnO_4 , H_2O , reflux 4hours; ii) BH_3 1M, THF; iii) SOCl_2 ; iv) N-Boc-ethylenediamine, K_2CO_3 /acetonitrile, reflux; v) HCl 1 M; vi) DO2A-bis tert butyl ester, DIPEA, HBTU; vii) $\text{TFA}/\text{H}_2\text{O}$ (20/80); viii) $\text{GdCl}_3/\text{H}_2\text{O}$.

Synthesis of Pyrazole-3-carboxylic acid (2)

3-methylpyrazole (**1**) (5.24 g, 63.7mmol) was dissolved in 200 mL Milli Q grade water followed by slow addition of 75 mL aqueous KMnO_4 (22g, 2.2 equiv.) at room temperature. Mixture was refluxed for 4 hours, a color change from purple to brown was observed indicating the completion of the oxidation process. Reaction mixture was cooled down to room temperature and then filtered. The pH of the filtrate was decreased from 10.6 to 1.7 by adding concentrated HCl . A white solid precipitated upon acidification of the filtrate. The solid was filtered out and washed with cold concentrated HCl . 3.512 g of product were collected upon drying corresponding to a 49% yield.

^1H NMR (200 MHz, D_2O): δ 7.50 (1H, d, - C^5H -, $^3J_{\text{HH}}=2.6$), 6.56 (1H, d, - C^4H -, $^3J_{\text{HH}}=2.6$). $^{13}\text{C}\{^1\text{H}\}$ NMR (50 MHz, D_2O): 170.15 (-COOH), 146.44 (- C^3 -), 136.63(- C^5H -), 109.97 (- C^4H -). Anal. calcd. for $\text{C}_4\text{H}_4\text{N}_2\text{O}_2$: C, 42.86; H, 3.60; N, 24.99. Found: C 42.93, H 3.59, N 25.02

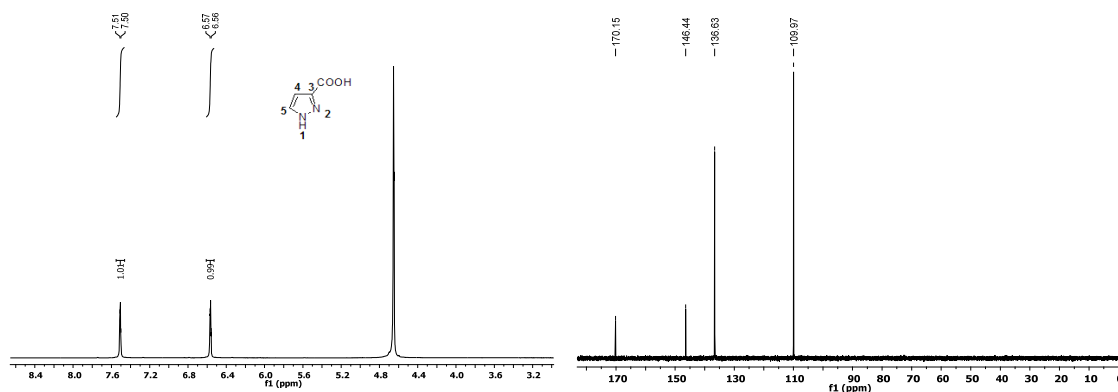


Figure S1. ^1H NMR (left) and $^{13}\text{C}\{^1\text{H}\}$ (right) NMR spectra of Pyrazole-3-carboxylic acid.

Synthesis of 3-(chloromethyl)pyrazole.HCl (4)

Pyrazole-3-carboxylic acid (**2**) (1.94g, 17.3 mmol) was dissolved in 40 mL of dry THF under N₂ followed by addition of BH₃ (64mL, 1M) in THF. Reaction mixture was stirred at room temperature for 2 hours, then at 40 °C for 1 hour and then it was allowed to cool down to room temperature. 50 mL of Milli Q grade water were slowly added and the resultant mixture was stirred overnight at room temperature. Solution was concentrated to 30 mL by rotary evaporation and residue was washed with dichloromethane 2x 20 mL. The aqueous phase was collected and concentrated to 5 mL and a white solid was observed. Solid was recrystallized, filtered and dried giving 3-(hydroxymethyl)pyrazole (**3**) quantitatively (1.70 g). The obtained solid is very hygroscopic and was used immediately without further characterization. 5 mL of neat thionyl chloride were added to (**3**) and mixture was stirred overnight at room temperature. 10 mL of Milli Q water were added to the reaction mixture and resultant solution was stirred for 1 hour to eliminate the excess of thionyl chloride. Solution was filtered out, the filtrate was collected and solvent was removed under high vacuum resulting in a transparent oil. The oil was dried further adding 20 mL of dichloromethane followed by its removal by rotary evaporation. This procedure was repeated 4 times giving a white solid that upon drying under high vacuum gave 2.467 g which correspond to 93.2% yield as for the HCl salt of the product.

¹H NMR (200 MHz, D₂O, DCl 0.1M): δ 7.81 (1H, d, -C⁵H-, ³J_{HH} = 2.6), 6.51 (1H, d, -C⁴H-, ³J_{HH} = 2.6). ¹³C{¹H}NMR (50 MHz, D₂O): 145.44 (-C³-), 134.11(-C⁵H-), 106.98 (-C⁴H-), 34.47 (-CH₂-OH). Anal. calcd. for C₄H₅ClN₂.HCl: C, 31.40; H, 3.95; N, 18.31. Found: C 34.51, H 3.97, N 18.34.

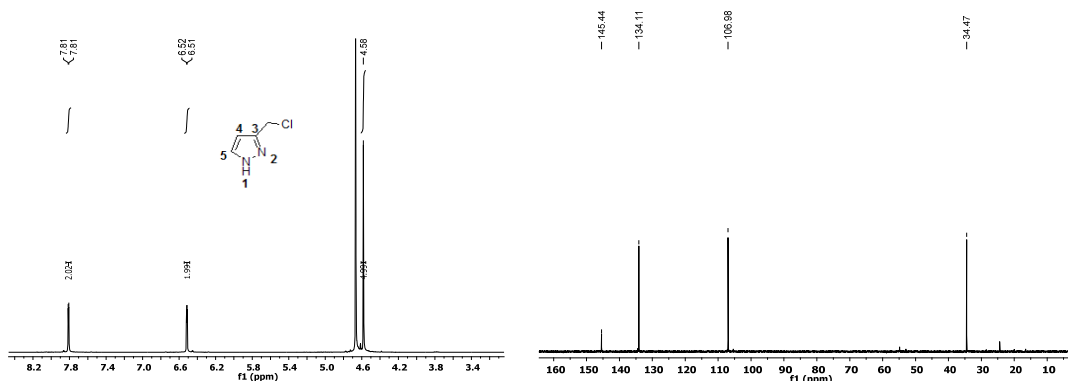


Figure S2. ¹H NMR (left) and ¹³C{¹H} (right) NMR spectra of 3-(chloromethyl)pyrazole.

Synthesis of N-(2-pyrazolylmethyl) ethylene diamine (5)

N-Boc-ethylenediamine (430 mg, 2.684 mmol) was dissolved in 40 mL of acetonitrile under N₂ atmosphere followed by addition of 3-chloromethylenepyrazole (914 mg, 2 equiv.) and K₂CO₃ (2.967 mg, 8 equiv.). Resultant mixture was refluxed for 52 hours under N₂. Mixture was filtered and the solvent was removed from the filtrate under rotary evaporation. Product was purified by HPLC (isocratic 65%/35% water/acetonitrile containing 0.1%/0.08% TFA, Rt = 6.34 min) which after liophilization gave 694 mg of a white solid (81%) which corresponded to the trifluoroacetate salt of N-Boc-N'-(2-pyrazolylmethyl) ethylene diamine. ¹H NMR (200 MHz, D₂O, DCl 0.1M): δ 7.58 (2H, d, -C⁵H-, ³J_{HH} = 2.3), 6.35 (2H, d, -C⁴H-, ³J_{HH} = 2.3), 4.21 (4H, s, -CH₂-pyrazole), 3.17 (2H, t, Boc-NH-CH₂-, ³J_{HH} = 5.7), 3.03 (2H, bs, -CH₂-N-), 1.11 (9H, s, CH₃-). ¹³C{¹H}NMR (50 MHz, D₂O): 158.15 (-CO-), 139.87 (-

C³⁻), 131.93 (-C⁵H-), 107.60 (-C⁴H-), 81.69 (-C(CH₃)₃), 52.35 (Boc-NH-CH₂-), 49.93 (-CH₂-pyrazole), 29.68 (-CH₂-N-), 27.57 (-CH₃). (MALDI-TOF+) m/z = 321.39 (100%) [M+H]⁺ (calcd. 321.39), an appropriate isotope pattern was observed.

N-Boc-N'-(2-pyridyl-methyl) ethylene diamine (694 mg) was dissolved in 50 mL of HCl 1 M. Resultant solution was stirred overnight. Product was freeze dried giving 631 mg of a very hygroscopic white solid which corresponded to the 2HCl salt of N-(2-pyrazolylmethyl) ethylene diamine. ¹H NMR (200 MHz, D₂O): δ 7.70 (2H, d, -C⁵H-, ³J_{HH} = 2.3), 6.46 (2H, d, -C⁴H-, ³J_{HH} = 2.3), 4.29 (4H, s, -CH₂-pyrazole), 3.27 (4H, bs, -CH₂-). ¹³C{¹H}NMR (50 MHz, D₂O): 140.48 (-C³⁻), 132.23 (-C⁵H-), 107.69 (-C⁴H-), 49.95 (-CH₂-pyrazole), 48.94(-CH₂-N-CH₂-pyrazole), 34.36 (-CH₂-NH₂). (MALDI-TOF+) m/z = 221.41 (100%) [M+H]⁺ (calcd. 221.27), an appropriate isotope pattern was observed. Anal. calcd. for C₁₀H₁₆N₆·2HCl: C, 40.96; H, 6.19; N, 28.66. Found: C 41.05, H 6.25, N 28.87.

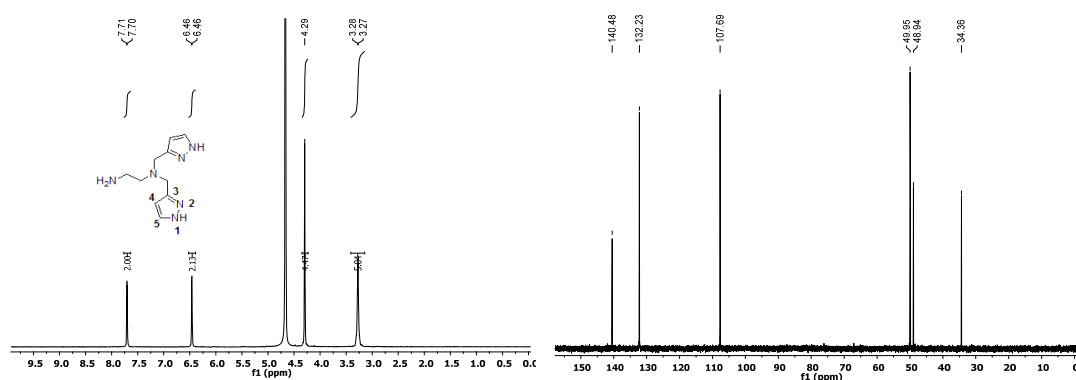


Figure S3. ¹H NMR (left) and ¹³C{¹H} (right) NMR spectra of N-(2-pyrazolylmethyl) ethylene diamine.

Synthesis of 1,4,7,10-Tetraazacyclododecane-1,4-bis(N,N-bis(2-pyrazolylmethyl) aminoethyleneacetamide)-7,10-acetic acid, DOTAdiBPYREN

1,4,7,10-Tetraazacyclododecane-1,4-bis(*tert*-butyl acetate)-7,10-acetic acid (DOTA-bis-*tert* butyl ester) (530 mg, 1.025 mmol), HBTU (808 mg, 2.13 mmol) and 200 μL of DIPEA were dissolved in 5 mL of DMF and the resultant yellow solution was stirred for 5 min at room temperature. The previous mixture was added to a 5 mL DMF solution containing N-(2-pyrazolylmethyl) ethylene diamine. 2HCl 631 mg (2.15 mmol) and 2.5 mL (26.6 mmol) of DIPEA. The resultant mixture was stirred overnight at room temperature. Solvent was removed under vacuum while heating at 50°C. 20 mL of dichloromethane were added to the residue and the mixture was washed with a 0.15 mM NaOH aqueous solution and the organic phase was collected. The basic washing was repeated 4x 25 mL, followed by one washing with 25 mL of Milli Q grade water. The organic phase was collected, dried with anhydrous Na₂SO₄, filtered and the solvent was removed by rotary evaporation. Product was purified by HPLC (H₂O/ACN 83/17 containing 0.1%/0.08% TFA as the eluent under isocratic conditions, R_t = 9.08 min). The fraction containing the compound was freeze-dried giving 368 mg of product (39%). ¹H NMR (200 MHz, D₂O): δ 7.63 (4H, d, -C⁵H-, ³J_{HH} = 2.2), 6.39 (4H, d, -C⁴H-, ³J_{HH} = 2.2), 4.28 (8H, s, -CH₂-pyrazole), 3.90 (4H, bs, -CH₂-COOtBu), 3.46 (4H, bs, -CH₂-CON), 3.46-3.25 (16H, bs, -CH₂-), 2.86-2.75 (8H, bs, -CH₂-CH₂-N(CH₂-Pyrazole)), 1.05 (18H, s, -CH₃). ¹³C{¹H}NMR (50 MHz, D₂O): 171.30 (-CONH-), 166.56 (-COO), 139.80 (-C³⁻), 131.91 (-C⁵H-), 107.69 (-C⁴H-), 82.54 (-C(CH₃)₃),

58.86 (-CH₂COO), 53.70 (-CH₂CONH), 52.01 (-CH₂-, cyclen), 51.64 (-CH₂-pyrazole), 49.63 (-CH₂-N-CH₂-pyrazole), 48.01(-CH₂-, cyclen), 34.49 (-CH₂-NHCO-), 27.28 (-CH₃). (MALDI-TOF+) m/z = 921.80 (100%) [M+H]⁺ (calcd. 922.15); 866.74 (22%) [M+H-*t*Bu]⁺ (calcd. 866.04), 809.79 (18%) [M+H-2*t*Bu]⁺ (calcd. 809.93), an appropriate isotope pattern was observed.

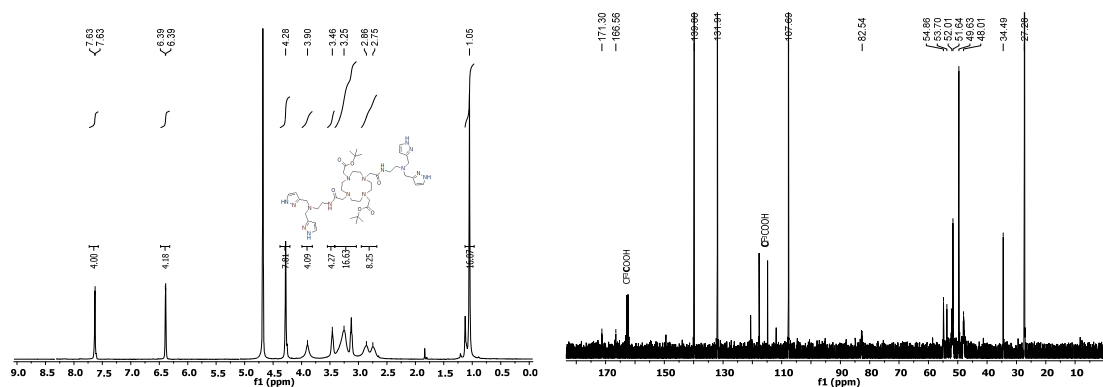


Figure S4. ¹H NMR (left) and ¹³C{¹H} (right) NMR spectra of DOTA-diBPYREN-bis-*tert*-butyl ester.

1,4,7,10-Tetraazacyclododecane-1,4-bis(N,N-bis(2-pyrazolylmethyl) aminoethyleneacetamide)-7,10-bis(*tert*-butyl acetate) (368 mg) was dissolved in TFA 95% in Milli Q grade water and stirred at room temperature for 2 hours. Solvent was removed under a gentle N₂ flow and residue was dissolved in Milli Q water and then it was freeze-dried giving 262 mg of the ligand as a white solid of the bis-trifluoroacetate salt. ¹H NMR (200 MHz, D₂O): δ 7.66 (d, -C³H- (pyrazole isomer), ³J_{HH} = 2.43), 7.60 (4H, d, -C⁵H-, ³J_{HH} = 2.40), 6.42 (d, -C⁵H- (pyrazole isomer), ³J_{HH} = 2.43), 6.35 (4H, d, -C⁴H-, ³J_{HH} = 2.40), 4.28 (8H, s, -CH₂-pyrazole), 3.90 (4H, bs, -CH₂-COOH), 3.46 (4H, bs, -CH₂-CON), 3.46-3.25 (16H, bs, -CH₂-), 2.86-2.75 (8H, bs, -CH₂-CH₂-N(CH₂-Pyrazole)). ¹³C{¹H}NMR (50 MHz, D₂O): 171.30 (-CONH-), 166.56 (-COO), 139.80 (-C³-), 131.91 (-C⁵H-), 107.69 (-C⁴H-), 82.54 (-C(CH₃)₃), 58.86 (-CH₂COO), 53.70 (-CH₂CONH), 52.01 (-CH₂-, cyclen), 51.64 (-CH₂-pyrazole), 49.63 (-CH₂-N-CH₂-pyrazole), 48.01(-CH₂-, cyclen), 34.49 (-CH₂-NHCO-), 27.28 (-CH₃). m/z (MALDI-TOF+) m/z = 921.80 (100%) [M+H]⁺ (calcd. 922.15), an appropriate isotope pattern was observed. Anal. calcd. for C₃₆H₅₆N₁₆O₆.2CF₃COOH: C, 46.33; H, 5.64; N, 21.61. Found: C 47.02, H 5.69, N 21.89.

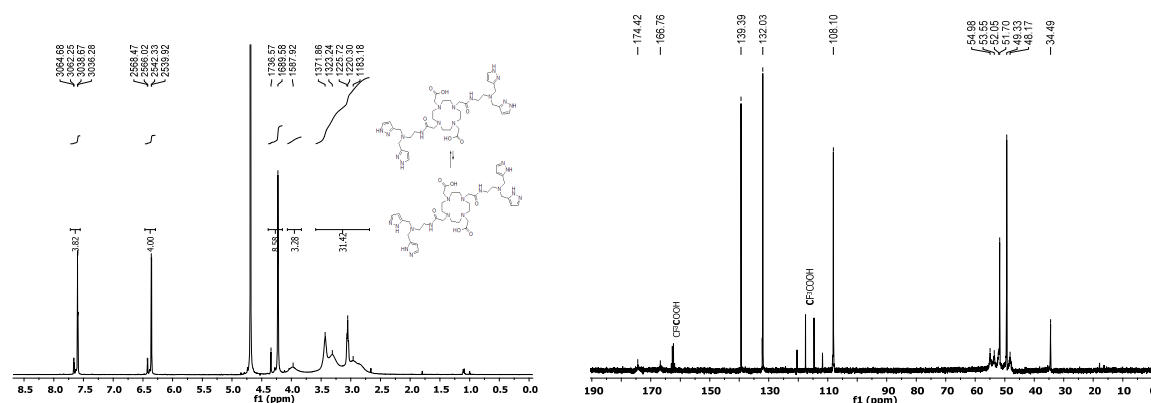


Figure S5. ¹H NMR (left) and ¹³C{¹H} (right) NMR spectra of DOTA-diBPYREN.

Synthesis of Gd³⁺DOTA-diBPYREN (6)

DOTA-diBPYREN (86 mg, 0.083 mmol) was dissolved in MilliQ grade H₂O and the pH adjusted to 6.5 with 1 M NaOH. GdCl₃•6H₂O (0.085 mmol) dissolved in water was slowly added in slight excess. The pH of the solution was maintained between 6 and 6.5 during addition. The resultant solution was stirred at room temperature for several hours and pH adjusted close to 6.5 as needed. Unreacted Gd³⁺ was precipitated as Gd(OH)₃ after the addition of 1 M NaOH and solution was filtered through a teflon 0.22μ filter. Gd content was assessed by ICP-OES and this was used to determine the amount of unsalted GdDOTA-diBPYREN (95.8% yield). The absence of free Gd was determined with the Xylenol Orange test.² The absence of free ligand was determined via analytical HPLC using aqueous (NH₄)₂CO₃ 5 mM pH 8/MeOH (50/50) as eluent.

as the eluant (MALDI-TOF+) m/z = 963.45 (100%) [M+H]⁺ (calcd. 964.17); 985.39 (37%) [M+Na]⁺ (calcd. 988.18);

3. MALDI-TOF MS spectra of a solution containing GdDOTA-diBPYREN and Zn²⁺

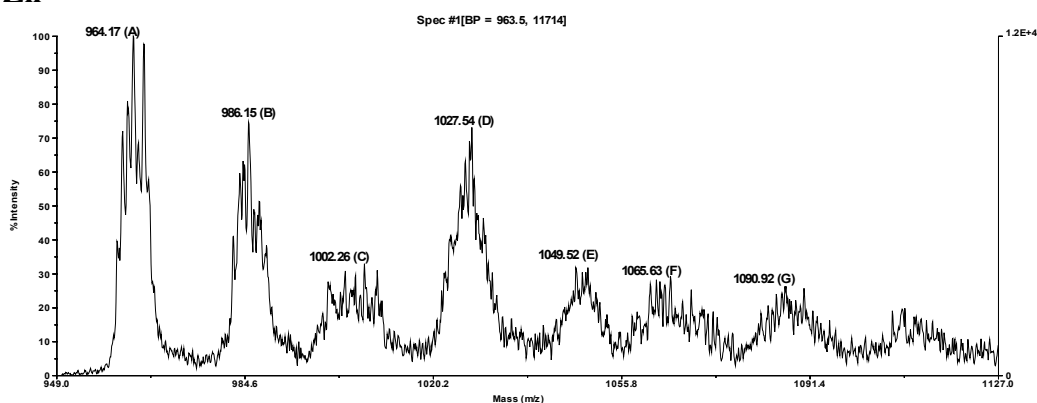


Figure S6. MALDI-TOF MS spectrum of a solution containing GdDOTA-diBPYREN and Zn²⁺. Shown values on top of each set of peaks are the calculated m/z for M⁺ species A, B, C, D, E, F and G which are depicted in Fig. S7.

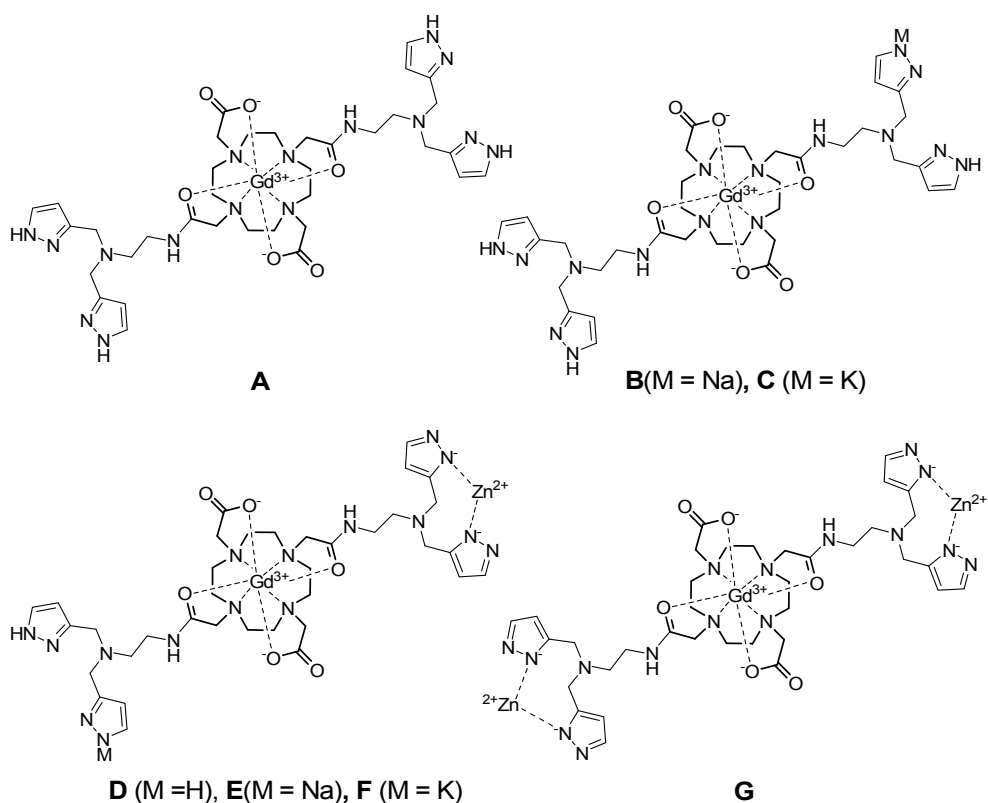
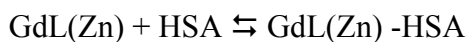


Figure S7. Proposed structures that correspond to the signals observed in MALDI-TOF MS spectrum of Fig. S6.

4. Determination of GdDOTA- diBPYREN-HSA and GdDOTA-Zn₂diBPYREN-HSA dissociation constant by relaxometric experiments

Solid HSA was titrated by adding increasing amounts of a Tris buffer solution 0.1 M pH 7.6 first with GdDOTA-diBPYREN 1 mM and Zn²⁺ 2 mM and then without. Another titration was performed in the same buffer with only GdDOTA-diBPYREN 1 mM. The *T*₁ values were recorded in all cases. Association constant was determined considering the following equilibrium and equations (2) to (4):



$$K_D = \frac{[\text{GdL}(\text{Zn})][\text{HSA}]}{[\text{GdL}(\text{Zn})\text{-HSA}]} \quad (2)$$

$$\Delta R = R_{1,obs}^{\text{GdL}(\text{Zn}),\text{HSA}} - R_{1,d}^{\text{HSA}} \quad (3)$$

$$\Delta R = \frac{R_b [\text{HSA}]_0}{K_D + [\text{HSA}]_0} \quad (4)$$

Where:

K_D is the binding dissociation constant.

$[GdL(Zn)\text{-HSA}]$ is the concentration of GdDOTA-diBPYREN or GdDOTA-Zn₂diBPYREN bound to HSA.

$[GdLZn]$ is the concentration of unbound GdDOTA-diBPYREN or GdDOTA-Zn₂diBPYREN in solution.

$[HSA]$ is the concentration of free HSA in solution.

$[HSA]_O$ is the total concentration of HSA.

ΔR is the relaxation rate enhancement.

R_b is the maximum value that the relaxation rate enhancement can reach extrapolated to the scenario where all the contrast agent is bound to HSA.

$R_{1,obs}$ is the determined relaxation rate ($1/T_1$) of the contrast agent in buffer or in buffer with HSA.

$R_{1,d}$ is the diamagnetic relaxation rate of buffer with HSA.

HSA titration with GdDOTA-diBPYREN was fitted to a one site - Fit total and nonspecific binding model in GraphPad Prism (equation 4). Fitting parameteres are shown below in Table S1, where B_{max} is R_b , NS is the slope of nonspecific binding, background is the amount of nonspecific binding with no added competitor. and K_D is given in the same units as $[HSA]$.

Table S1. Fitting parameters for the HSA titration with GdDOTA-diBPYREN.

One site -- Total and nonspecific binding

Best-fit values

Bmax	3.916	
Kd	0.01069	
NS	0.7010	0.7010
Background	4.545	4.545
Std. Error		
Bmax	0.09108	
Kd	0.0008868	
NS	0.03717	0.03717
Background	0.08501	0.08501
95% Confidence Intervals		
Bmax	3.731 to 4.101	
Kd	0.008888 to 0.01249	
NS	0.6256 to 0.7764	0.6256 to 0.7764
Background	4.372 to 4.717	4.372 to 4.717
Goodness of Fit		
Degrees of Freedom		36
R square	0.9905	0.9905
Absolute Sum of Squares	0.5263	0.5263
Sy.x		0.1209
Constraints		
NS	NS is shared	
Background	Background is shared	
Number of points Analyzed		40

HSA titration with GdDOTA-Zn₂diBPYREN was fitted to a one site - specific binding model in GraphPad Prism (equation 4). Fitting parameters are shown in Table S2, where B_{max} is R_b and K_D is given in the same units as [HSA].

Table S2. Fitting parameters for the HSA titration with GdDOTA-Zn₂diBPYREN.

One site -- Specific binding	
Best-fit values	
Bmax	14.27
Kd	0.02940
Std. Error	
Bmax	0.2146
Kd	0.002260
95% Confidence Intervals	
Bmax	13.79 to 14.75
Kd	0.02437 to 0.03444
Goodness of Fit	
Degrees of Freedom	10
R square	0.9631
Absolute Sum of Squares	0.1249
Sy.x	0.1118
Number of points	
Analyzed	12

5. Fluorimetric determination Zn²⁺ binding dissociation constant to GdDOTA-diBPYREN

5.1 Saturation binding experiment

A series of solutions containing 10 uM ZnCl₂ in tris buffer 0.1 M pH 7.6 each with increasing concentrations of FluoZin-1 (Invitrogen); 0 uM , 2 uM, 4 uM , 6 uM, 8 uM, 10 uM, 12 uM, 14 uM , 16 uM, 18 uM, 20 uM and 24 uM were prepared by duplicate. After mixing all solutions were allowed to stabilize for 30 minutes at room temperature. The fluorescence of 100 µL aliquots of each of these samples was measured in duplicate in 96 well plates, the excitation and emission wavelengths used were 495 and 517 nm, respectively. The Zn²⁺ FluoZin-1 dissociation constant was determined by fitting the data to the following equation:

$$F = \frac{F_{\max} [FluoZin - 1]_O}{K_D + [FluoZin - 1]_O} \quad (5)$$

Where:

[FluoZin-1]_O is the total concentration of FluoZin-1.

K_D is the binding dissociation constant given in the same units as [FluoZin-1]_O.

F is the measured corrected fluorescence.

F_{max} is the maximum value that the fluorescence can reach extrapolated to the scenario where all the Zn²⁺ is bound to FluoZin-1.

Zn²⁺ titration with FluoZin-1 was fitted to a one site - specific binding model in GraphPad Prism (equation 5). Fitting parameters are shown in Table S3 and fitted data is shown in Fig. S8.

Table S3. Fitting parameters for the titration of Zn^{2+} with FluoZin-1.

One site -- Specific binding	
Best-fit values	
Bmax	4114
Kd	24.27
Std. Error	
Bmax	295.8
Kd	2.853
95% Confidence Intervals	
Bmax	3501 to 4728
Kd	18.36 to 30.19
Goodness of Fit	
Degrees of Freedom	22
R square	0.9895
Absolute Sum of Squares	98800
Sy.x	67.01
Number of points	
Analyzed	24

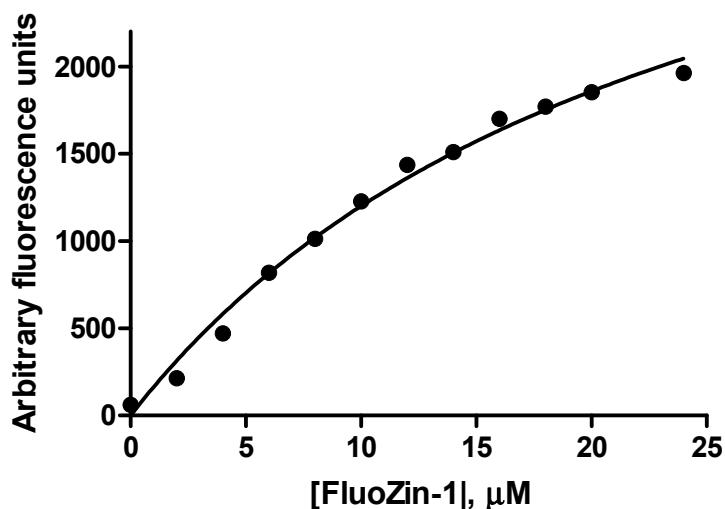


Figure S8. Saturation binding curve for determination of Zn^{2+} binding dissociation constant to FluoZin-1..

5.2 Competition binding experiment

A solution containing 10 μM of FluoZin-1, 10 μM ZnCl_2 and 200 μM GdDOTA-diBPYREN in buffer was prepared. Aliquots of this solution were 3-fold diluted serially by addition of a buffered solution which contained the same concentration of FluoZin-1 and ZnCl_2 but without agent. This gave fourteen samples of different concentrations of agent (differing by a factor of 3), but all containing the same concentration of FluoZin-1 and ZnCl_2 , samples were prepared by duplicate. The fluorescence of 100 μL aliquots of each of these samples was measured in duplicate in 96 well plates. In a similar manner solutions containing agent, ZnCl_2 but no fluorescent probe were prepared and fluorescence measured by duplicate in order to account for any signal coming from the agent interacting with Zn^{2+} . The excitation and emission wavelengths used were 495

and 517 nm, respectively. The Zn dissociation constant was determined by fitting the data to the following equations:

$$F = F_{\min} + \frac{F_{\max} - F_{\min}}{1 + 10^{(\log[\text{Competitor}]_o - \log IC50)}} \quad (6)$$

$$IC50 = K_D' \left(1 + \frac{[FP]_o}{K_D} \right) \quad (7)$$

Where:

F is the measured corrected fluorescence.

F_{\min} is the minimal fluorescence observed when Zn^{2+} is all bound to the competitor. For this experiment it was set to the value of the background signal, that is a solution without fluorescent dye which was arbitrarily assigned a $[Competitor]_o$ of 100 mM. FluoZin-1 does not show significant signal at 517 nm in absence of Zn^{2+} .

F_{\max} corresponds to the fluorescence in absence of competitor.

$[Competitor]_o$ is twice the total concentration of the competitor, that is GdDOTA-diBPYREN.

$IC50$ corresponds to the concentration of competitor that decreases by 50% the $\%F_{\max}$.

K_D is the dissociation constant of the competitor.

$[FP]_o$ is the total concentration of the fluorescent probe in nM.

K_D is the dissociation constant of the fluorescent probe and Zn^{2+} in nM. For FluoZin-1 a K_D of 24.3 μ M was determined.

Competition data was fitted to a one site – Fit K_i model in GraphPad Prism (equations 6 and 7). Fitted data is shown in Figure S9 and fitted parameteres are shown in Table S4, where bottom is F_{\min} , top is F_{\max} , HotNM is $[FP]_o$, HotKdM is K_D both in nM units and K_D is given in the same units as of $[Competitor]_o$.

Table S4. Fitting parameters for the titration of Zn^{2+} and FluoZin-1 with GdDOTAdiBPYREN.

One site - Fit K_i

Best-fit values

log K_i	2.578
HotNM	= 10000
HotKdNM	= 24270
Bottom	84.98
Top	1223
K_i	378.6

Std. Error

log K_i	0.05263
Bottom	35.87
Top	10.15

95% Confidence Intervals

log K_i	2.471 to 2.686
Bottom	11.62 to 158.3
Top	1202 to 1244
K_i	295.5 to 485.1

Goodness of Fit

Degrees of Freedom 29

R square 0.9734

Absolute Sum of Squares 73002

Sy.x 50.17

Constraints

HotNM HotNM = 10000

HotKdNM HotKdNM = 24270

Number of points

Analyzed 32

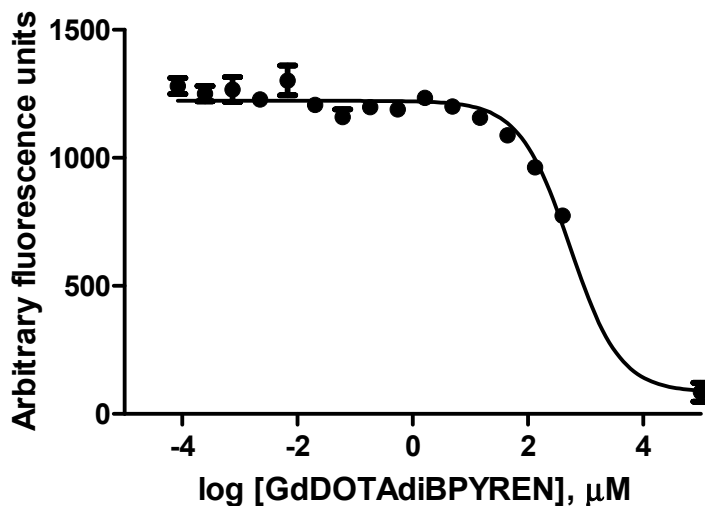


Figure S9. Competition binding curve for determination of Zn^{2+} binding dissociation constant to GdDOTAdiBPYREN.

6. MRI of Mice

Twenty four hour fasted male C57/blk6 mice were anesthetized with isoflurane (1-2% in medical oxygen) and probes were placed on the skin for monitoring temperature and on the chest for monitoring respiration. Anesthetized mice were positioned in the bore of a 9.4T magnet and kept warm using a stream of temperature-regulated air. 150 μ L of a 20% (w/v) glucose solution (\sim 30 mg total typical) was injected intraperitoneally (saline in controls) while 50 μ L of a 50 mM stock solution of GdDOTA-diBPYREN (ProhanceTM in controls) was injected via a tail vein catheter. The T1-weighted images were collected using a FSEMS sequence with the following parameters: TR = 89.03 ms; effective echo time (TE) = 11.21 ms; FOV 30x30 mm², data matrix = 256x256, averaging = 3, slice = 1 mm, number of slices = 6, gap = 0; ETL=1, kzero = 1. No gating was employed. The averaging and lowered respiration under anesthesia allowed for the true TR in the regular fsems sequence and gave acceptable CNR and SNR levels. The i.p. injection of glucose (t = 0 min) was done 10 minutes prior to i.v. injection of GdDOTAdiBPYREN or ProhanceTM via tail vein catheter. A series of images were obtained between this glucose stimulation and agent injection (total multi-slice acquisition time \sim 2 minutes).

7. References

- 1 L. M. De Leon-Rodriguez, Z. Kovacs, A. C. Esqueda-Oliva and A. D. Miranda-Olvera, *Tetrahedron Lett.* 2006, **47**, 6937.
- 2 A. Barge, G. Cravotto, E. Gianolio and F. Fedeli, *Contrast Media Mol. Imaging* 2006, **1**, 184.