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Synthesis, Relaxation Properties and *In vivo* Assessment of Carborane-GdDOTA-Monoamide Conjugate as MRI Blood Pool Contrast Agent

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Abstract

The synthesis, relaxivity measurements and *in vivo* assessment of a carborane-GdDOTA-monoamide (**CB-GdDOTA-MA**) amphiphilic conjugate as blood pool contrast agent (BPCA) is reported. This BPCA exhibited excellent binding (87.4%) with human serum albumin (HSA) and showed higher relaxivity value (r_1 = 6.8 $mM^{-1}s^{-1}$, 7 T) as compared to the clinically used BPCA, MS-325 (r_1 = 5.1 $mM^{-1}s^{-1}$, 9.4 T) in PBS. The blood pool contrast enhancement (CE) capability of **CB-GdDOTA-MA** was evaluated by performing MR angiography (MRA) in CF1 mice (n = 4) at a Gd dose of 0.1 mmol/kg body weights. The significant CE of blood vessels was persisted for about 3-4 min post-injection (p.i.) and quickly diminishes over time. The significant CE of the bladder for up to 3 h p.i indicated the renal system is the primary clearance pathway for **CB-GdDOTA-MA**. However, the CE of liver tissues and intestine (up to 24 h p.i.) is a suggestive of significant hepatic uptake of the **CB-GdDOTA-MA**.

Introduction

During magnetic resonance imaging (MRI), contrast agents (CAs) are commonly utilized due to their ability to enhance the longitudinal relaxation rate ($R1 = 1/T1$) of the protons of water molecules.[1-2] Clinically used low molecular weight (LMW) CAs e.g. Gd(DTPA) (DTPA = diethylenetriaminepentaacetic acid) and Gd(DOTA) (DOTA = 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) complexes have a relatively low relaxivity (r_1) and are non-targeted.[3-4] However**,** several successful strategies have been reported to increase the r_1 as well as to target the CAs to the region of interests.[5-6] Amongst all, the most promising strategies are protein-targeted MRI CAs, such as MS-325 (Fig. 1, **A**).[7] MS-325 is an amphiphilic derivative of

Gd(DTPA) approved for clinical use as blood pool contrast agent (BPCA). It binds strongly and reversibly to the blood protein, human serum albumin (HSA). The binding of MS-325 with HSA (size 67 kD) provides both the pharmacodynamics and pharmacokinetic effect; first, it reduces its molecular tumbling (τ_R) significantly which results in high r_1 in HSA particularly at intermediate magnetic field strengths, second, the binding with HSA reduces its glomerular filtration thus increases its plasma half-life compared to parent Gd(DTPA) chelate.[8] In general, the acyclic polycarboxylate ligand such as DTPA is known to be kinetically less stable as compared to cyclic polycarboxylate ligand such as DOTA, therefore the clinical use of DTPA ligand based CAs has been limited in the patients with renal dysfunctions due to risk of nephrogenic systemic fibrosis or NSF.[9] Nonetheless, the clinical success of MS-325 has inspired the development of several other BPCAs, however, none of them has made to the clinics yet. Majority of the BPCAs reported so far are bifunctional molecules comprising a protein-binding moiety (hydrophobic component) and a metal chelate.[10-12] It is well established that hydrophobic component represented by aromatic moieties in BPCAs is responsible for the generation of noncovalent interactions between the corresponding Gd^{3+} chelate and HSA.

The *closo*-$C_2B_{10}H_{12}$ (CB) clusters occupy a volume similar to adamantane and are a little larger (diameter 5.3 Å) than a rotating benzene ring (diameter 4.7 Å). The unique characteristics of CB such as resistance to catabolism, strong hydrophobicity, structural stability, ease of substitution, and delocalized bonding allows the CB clusters bioisosteric replacement for phenyl rings as rigid scaffolding in bioactive molecules and pharmacological agents. This phenyl-mimetic geometry and aromaticity of CB has already been applied in the synthesis of CB analogues of known drugs to enhance hydrophobic interactions between pharmaceuticals and their targets.[13-14] Therefore, we envisage the strong hydrophobic phenyl-mimetic character of CB pharmacophore could be used as an HSA binding moiety in bifunctional BPCA. We also expect the Carborane-GdDOTA-monoamide (**CB-GdDOTA-MA**) conjugate (Fig. 1, **B**) could be advantageous as BPCA due to the proven clinical safety of DOTA ligand. Herein, we describe the synthesis, relaxivities and *in vivo* MR angiography (MRA) results of a novel **CB-GdDOTA-MA** conjugate.

Earlier, the CB-GdDTPA conjugate has been reported as dual labelled probe for imaging (MRI) guided Boron Neutron Capture Therapy (BNCT) of cancers.[15] However; later it was found that this compound was not suitable for BNCT due to poor tumor accumulation.[16] Moreover, the

recent work on lipid derivatives of CB-DOTA conjugates form Aime and co-workers further emphasizes imaging (MRI) guided BNCT. Such lipophilic conjugates are designed to form nanoparticles (micelles or liposomes) and deliver sufficient boron atoms to the tumor via enhanced permeability and retention (EPR) effect.[17-19]

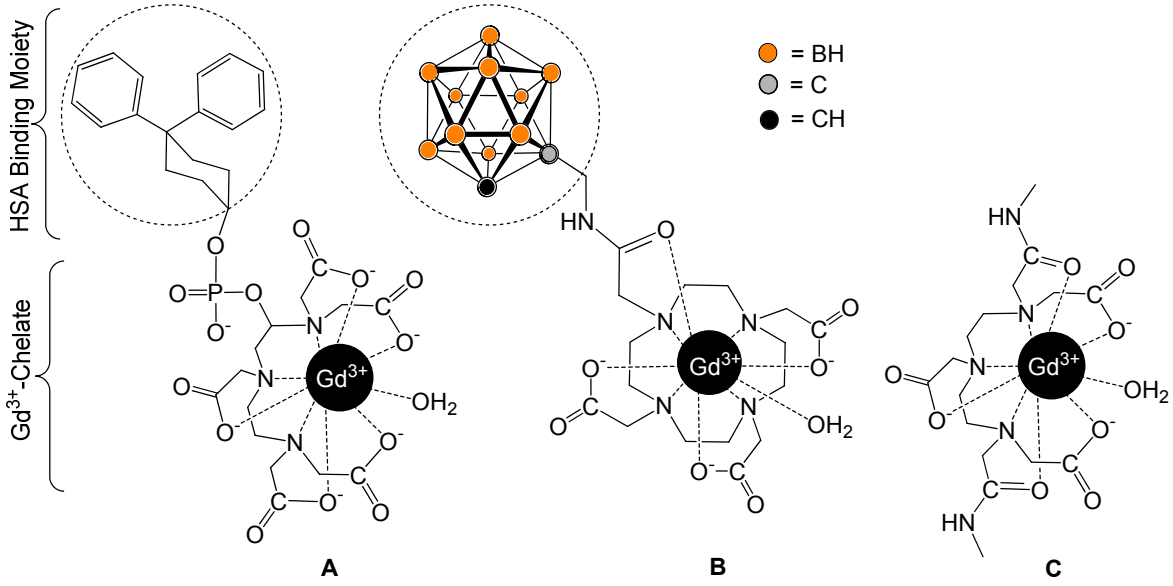


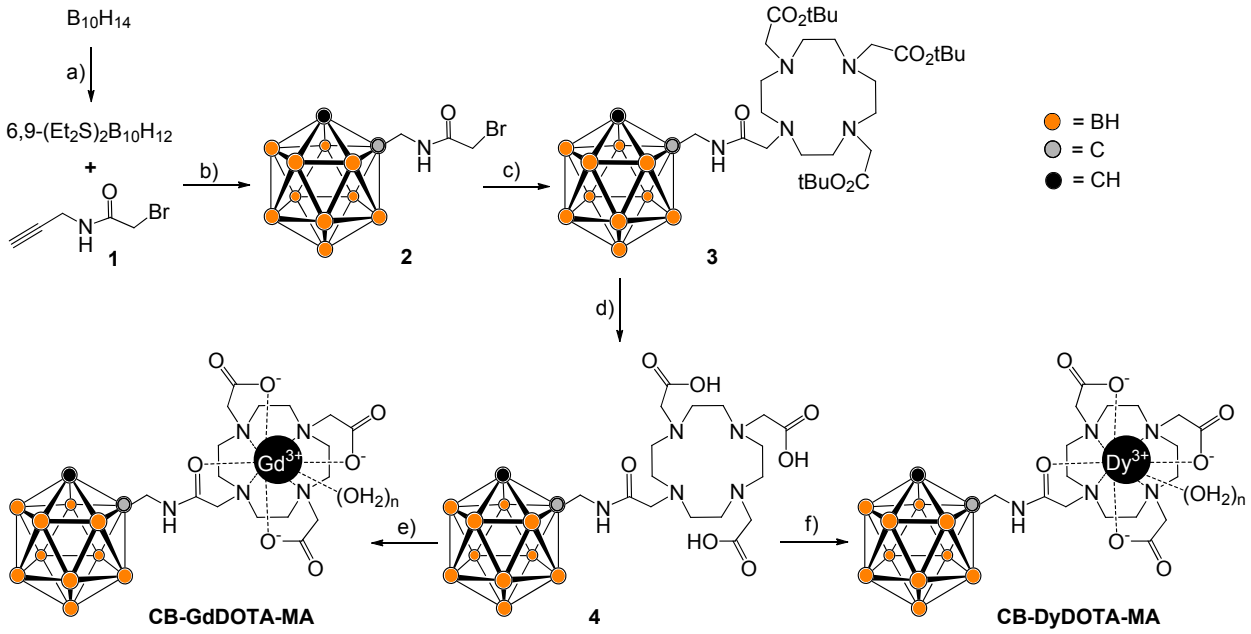
Fig. 1 Structure of a clinically approved BPCA MS-325 (**A**), **CB-GdDOTA-MA** (**B**) described herein and a non-targeted CA Omniscan (**C**).

Results and discussion

Synthesis of CB-GdDOTA-MA

Our efforts to prepare **CB-GdDOTA-MA** started with the synthesis of alkyne **1** in 89% yield by reacting propargylamine with bromoacetyl bromide. Next, the diethylsulfide (Et_2S) complex $B_{10}H_{12}(Et_2S)_2$ was obtained from the reaction of decaborane ($B_{10}H_{14}$) with 2 eq. of Et_2S in toluene at 85 °C for 2 h and reacted *in-situ* with alkyne **1** to obtain CB derivative **2** in 41% yield after silica-gel column chromatography. The CB derivative **2** was reacted with DO3A-*t-Bu*-ester to obtain **3** in 60% yield. The *t-Bu*-ester groups on **3** were deprotected using formic acid to obtain **4** in 82% yield. The final gadolinium complex, **CB-GdDOTA-MA** was prepared in 73% yield through the reaction of **4** with $GdCl_3.6H_2O$. Similarly, the dysprosium complex, **CB-DyDOTA-MA** was prepared in 68% yield through the reaction of **4** with $DyCl_3.6H_2O$ (Scheme 1). All the intermediate compounds were characterized using various spectroscopic techniques i.e. NMR,

HRMS analysis. The final **CB-GdDOTA-MA** conjugate was characterized using IR and HRMS analysis and the purity was established using inductively coupled plasma optical emission spectroscopy (ICP-OES) and HPLC analysis (ESI, Fig. S1 & S2). The hydration number (average q = 1.2) was determined via Dy^{3+}-ion induced ^{17}O NMR shift method (Electronic supplementary information or ESI, Fig. S3).[20] The Gd^{3+} ion conc. of the solutions used for relaxivity measurements and *in vivo* MRA was determined using ICP-OES.



Scheme 1 Synthesis of **CB-GdDOTA-MA** and **CB-DyDOTA-MA**. Reagents and conditions: a) Et_2S, Toluene, 85 °C, 2 h. b) Acetonitrile, 65 °C, 12 h, 41%. c) DO3A-*t*-Bu-ester, *N, N*-diisopropylethylamine, 85 °C for 12 h, 60%. d) Formic acid, 60 °C, 12 h, 82%. e) $GdCl_3.6H_2O$, H_2O-MeOH (1:1), pH 6.5, RT, 12 h, 73%. f) $DyCl_3.6H_2O$, H_2O-MeOH (1:1), pH 6.5, RT, 12 h, 68%.

Binding studies

The percentage (%) binding of **CB-GdDOTA-MA** (0.1 mM) with HSA (4.5% w/v = 0.67 mM) in PBS at 7.4 pH and 37 °C was determined using the ICP-OES analysis method reported by Caravan and co-workers.[8] As anticipated, the % binding of the **CB-GdDOTA-MA** (87.4%) with HSA was found to be similar to that of MS-325 (88%) reported in the literature.[8] The non-covalent serum binding ability of the CB moiety has also been confirmed by Tatham et al.[21]

Relaxivity Studies

The longitudinal and transverse relaxation rate (R_1 and R_2 respectively) for **CB-GdDOTA-MA** was obtained at various Gd^{3+} ion conc. in PBS at 7.4 pH and 23 °C at 7 T (Fig. 2). The final r_1 and r_2 values were obtained by linear fitting of relaxation rates against conc. for each sample and compared with clinically used CAs, MS-325 and Omniscan (Table 1). The r_1 value for **CB-GdDOTA-MA** in PBS (r_1 = 6.8 $mM^{-1}s^{-1}$) was found to be slightly higher than most of the clinically used non-targeted LMW CAs, including Omniscan (r_1 = 4.5 $mM^{-1}s^{-1}$ in PBS) and HSA targeted BPCA, MS-325 (r_1 = 5.1 $mM^{-1}s^{-1}$ in PBS at 9.4 T).[22] The higher relaxivity of the **CB-GdDOTA-MA** as compared to the Omniscan and MS-325 could be ascribed to the unexpectedly higher q value of the complex (Average q = 1.2, a mixture of q = 1 and q = 2 species, ESI, Fig. S3). Although, the r_2 value for **CB-GdDOTA-MA** in PBS (r_2 = 10.0 $mM^{-1}s^{-1}$ at 7 T) was found to be higher than non-targeted CA, Omniscan (r_2 = 5.4 $mM^{-1}s^{-1}$ at 7 T), however it was significantly lower when compared to the MS-325 (r_2 = 15.7 $mM^{-1}s^{-1}$ in PBS at 9.4 T). The lower r_2 value of **CB-GdDOTA-MA** as compared to the MS-325 could be attributed to the slower water exchange rate of the DOTA type ligands that severely limits the relaxivity of macromolecular CAs.[22-23]

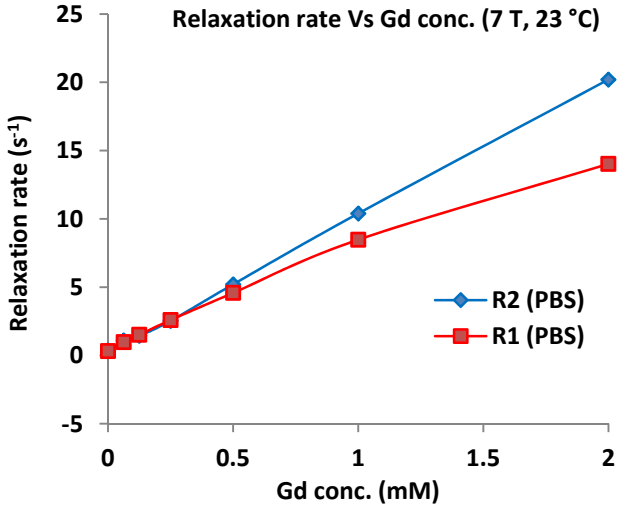


Fig. 2 Longitudinal and transverse relaxation rate (R_1 and R_2 respectively) for **CB-GdDOTA-MA** at various Gd^{3+} ion conc. in PBS at 7.4 pH and 23 °C at 7 T.

Table 1 Comparison of high field relaxivities (r_1 and r_2) of **CB-GdDOTA-MA** with clinically used CAs, MS-325 and Omniscan at 7.4 pH and 23 °C.

Compound	% Bound to 4.5% HSA	r_1 (PBS)	r_2 (PBS)
CB-GdDOTA-MA (7 T)	87.4	6.8 $\pm$ 0.3	10.0 $\pm$0.1
MS-325 (9.4 T)	88	5.1	15.7
Omniscan (7 T)	N/A	4.5 $\pm$ 0.08	5.4 $\pm$0.15

The T1-weighted MRI images of **CB-GdDOTA-MA** and Omniscan at various conc. in PBS are presented below (Fig. 3). The greater contrast enhancement for the **CB-GdDOTA-MA** as compared to the Omniscan is attributed to the higher relaxivity of the **CB-GdDOTA-MA** conjugate.

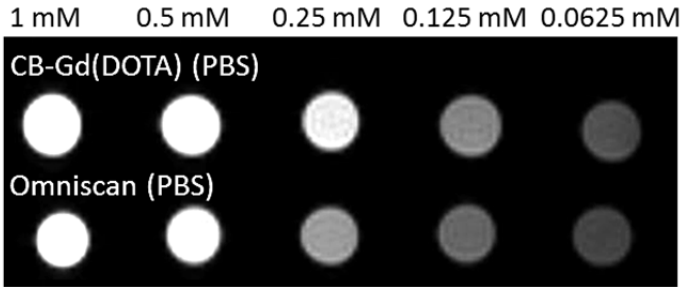


Fig. 3 T1-weighted MR images of **CB-GdDOTA-MA** and Omniscan at various Gd-conc. in PBS (7 T).

***In-vivo* MRA studies**

The blood pool contrast enhancement (CE) capability of **CB-GdDOTA-MA** was examined by performing MRA in CF1 mice (n = 4). The representative T1-weighted MRA scans (Fig. 4) show the significant CE of vascular system immediately after the injection of **CB-GdDOTA-MA** at a Gd dose of 0.1 mmol/kg body weights. Significant CE of blood vessels was persisted for about 3-4 min post-injection (p.i.) and quickly diminishes over time. The significant CE of the bladder for up to 3 h p.i indicates the renal system is the primary clearance pathway for **CB-GdDOTA-MA**. However, the CE of liver tissues and intestine (up to 24 h p.i.) is a suggestive of significant hepatic uptake of the **CB-GdDOTA-MA**.

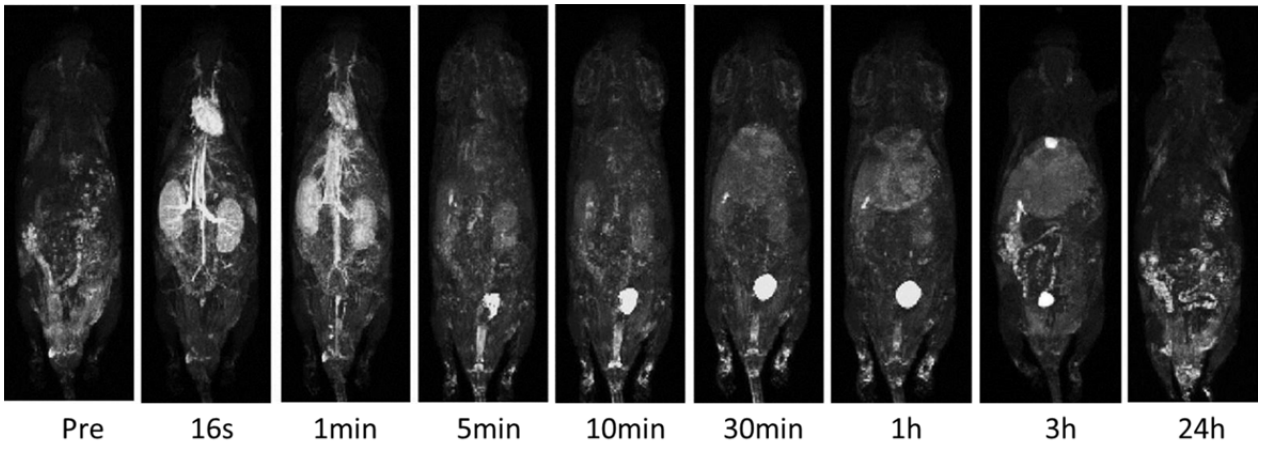


Fig. 4 Representative *in vivo* T1-weighted MRA scans of a mouse at various time points p.i. of **CB-GdDOTA-MA** with a Gd dose of 0.1 mmol/kg.

The CE ratios (CER) for various organs (Fig. 5) further confirms the significant (60%) CE in blood p.i. of **CB-GdDOTA-MA**. However, the vascular half-life of **CB-GdDOTA-MA** was ~8 min as compared to MS-325 (28.5 min in rodents). Unusually, the MS-325 has prolonged plasma half-life (18.5 h) in human as compared to rodents (28.5 min) due to its significant hepatic uptake in rodents.[24] Therefore, in spite of high HSA binding ex-vivo (87.4%), we were not surprised to see rapid plasma clearance of the **CB-GdDOTA-MA** due to the significant liver uptake which could reduce the plasma half-life significantly (Fig. 4 & 5). The MS-325 (Fig. 1, **A**) contains a bulky hydrophobic residue (two phenyls rings attached to a cyclohexyl moiety), therefore, it could be rationalized that more than one CB moiety per Gd-chelate may be required increase the plasma half-life for MRA application.

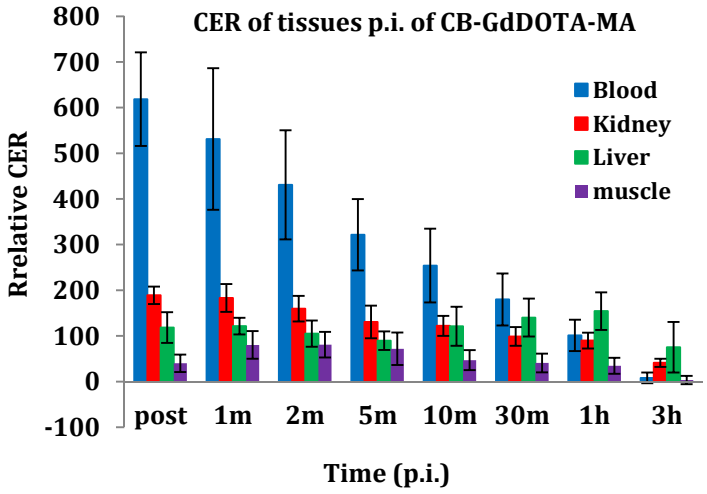


Fig. 5 CER profile for various organs of mice (n= 4) p.i. of **CB-GdDOTA-MA** with a Gd^{3+} dose of 0.1 mmol/kg.

Nonetheless, the mice injected with a similar dose of Omniscan (0.1 mmol/kg) did not show any vascular CE during MRA. Therefore, a 12 times higher dose (1.2 mmol/kg) of Omniscan was used to obtain measurable vascular CE. However, the significant CE lasted for only less than 1 min p.i. and quickly diminished over time (Fig. 6 and ESI, Fig. S4). The lower vascular CE by Omniscan even at a 12 times higher dose (1.2 mmol/kg) as compared to **CB-GdDOTA-MA** (0.1 mmol/kg) was expected due to lack of HSA binding ability.

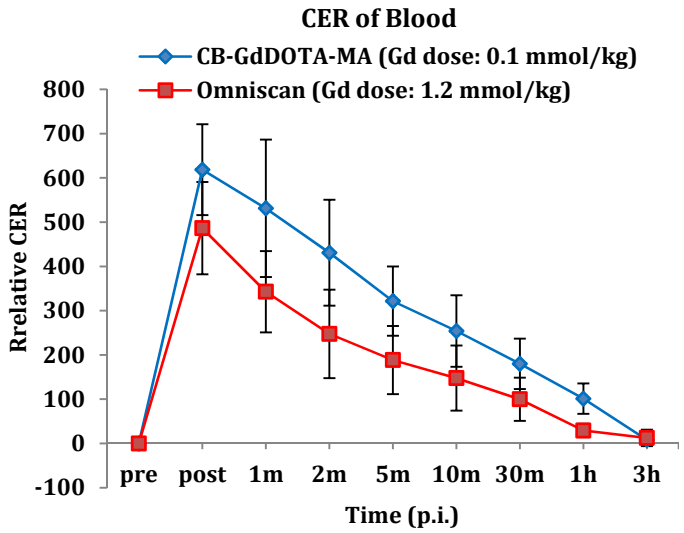


Fig. 6 CER profile for blood of mice (n = 4) p.i. of **CB-GdDOTA-MA** (Gd^{3+} dose of 0.1 mmol/kg) and Omniscan (Gd^{3+} dose of 1.2 mmol/kg).

After 24 h p.i., all mice were euthanized and blood, heart, lungs, liver, spleen, kidneys, brain, and muscle tissues were collected and analyzed for the presence of Gd^{3+} ions using ICP-OES analysis. Only a negligible amount of Gd was retained by the tissues analyzed (Fig. 7), with liver, spleen and kidney being the highest (~1.5% of total Gd injected).

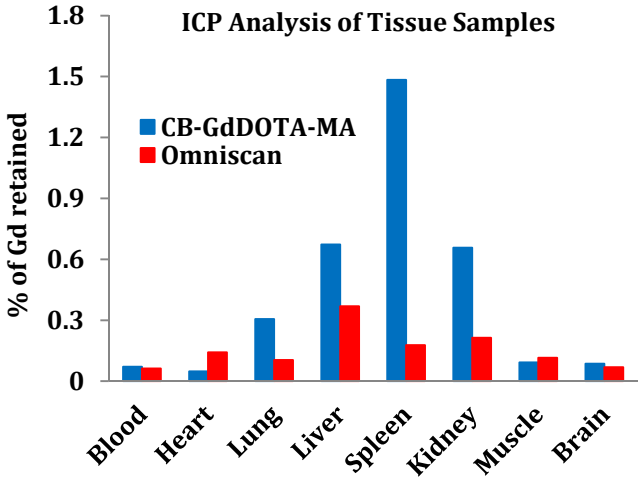


Fig. 7 ICP analysis of various tissue samples shows the percentage of total injected Gd^{3+} ions retained in various organs of mice (n = 4), 24 h p.i. of **CB-GdDOTA-MA** (Gd dose: 0.1 mmol/kg) and Omniscan (Gd dose: 1.2 mmol/kg).

Conclusions

In conclusions, we exploited the phenyl-mimetic hydrophobic characteristic of CB moiety towards the synthesis of a BPCA for magnetic resonance angiography application. The *ex-vivo* binding experiment suggest a strong non-covalent binding of **CB-GdDOTA-MA** with HSA. Likewise, the high field (7 T) r_1 values of **CB-GdDOTA-MA** were found to be comparable to the MS-325, a clinically used BPCA. However, *in vivo* MRA studies in mice using **CB-GdDOTA-MA** at a Gd dose of 0.1 mmol/kg body weight showed that the significant CE of vascular system persisted for about 3-4 min p.i. and quickly diminishes over time. The short plasma half-life of **CB-GdDOTA-MA** could possibly limit its application as BPCA and more than one CB moiety per Gd-chelate may be necessary.

Experimental procedures

General information

Common reagents and chromatographic solvents were obtained from commercial suppliers (VWR International, Sigma-Aldrich, and Fisher Scientific) and used without any further purification. DO3A-*t*-Bu-ester was purchased from Macrocyclics. NMR spectra were recorded on Bruker Avance 400 and 500 MHz spectrometers. All NMR chemical shifts (δ, ppm) for ^{1}H and ^{13}C were referenced to residual solvent peaks. Boron NMR chemical shifts were externally

referenced to BF_3*Et_2O. The high-resolution mass spectrometry analysis was performed using an Applied Biosystems Mariner ESI-TOF. IR spectra were recorded on a Thermo Nicolet FT-IR spectrometer. MRI measurements were performed using a 7 Tesla Bruker AVANCE III BioSpec MRI system (Bruker BioSpin Inc., Billerica, MA) at 23 °C. The Gd conc. of samples used in MRI experiments were measured by inductively coupled plasma optical emission spectroscopy (ICP-OES) using a PekinElmer OptimaTM 7000 DV instrument. Analytical HPLC analysis was performed on Alliance Waters 2695.

Synthesis of 2-bromo-N-(prop-2-yn-1-yl)acetamide (1). Propargylamine (3.0 g, 54.5 mmol) was dissolved in 100 mL of dichloromethane (DCM) and 100 mL of saturated aqueous sodium bicarbonate ($NaHCO_3$) were added. The mixture was vigorously stirred at -10°C and bromoacetyl bromide (16.5 g, 81.7 mmol) was added to it slowly. Reaction mixture was slowly allowed to warm to room temperature (RT). After 3 h the reaction mixture was concentrated partially to remove organic solvent and then poured into 100 mL of water. The reaction mixture was then extracted with ethylacetate (EtOAc) (2 x 200 mL). The organic layer was separated and washed with saturated aqueous $NaHCO_3$ (100 mL), 5% HCl-water (100 mL), and brine (100 mL). Drying over sodium sulfate (Na_2SO_4) and removal of the solvent under reduced pressure afforded the pure product as white solid. Yield: 8.6 g (89%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.73 (brs, 1H), 4.08 (dd, 2H, J = 2.5 & 5.5 Hz), 3.89 (s, 2H), 2.28 (t, 1H, J = 2.5 Hz). ^{13}C NMR (125 MHz, $CDCl_3$): δ 165.1, 78.5, 72.2, 29.9, 28.6. HRMS (m/z): Calcd for C_5H_6BrNO $[M+Na]^+$ 197.9525. Found 198.0366.

Synthesis of o-CB-2-bromoacetamide conjugate (2). Decaborane or $B_{10}H_{14}$ (0.25 g, 2.04 mmol) was dissolved in 20 mL of toluene and the resultant mixture was stirred at 85 °C. To this stirring mixture, diethyl sulfide or Et_2S (0.37 g, 4.08 mmol) was added and stirring was continued at 85 °C for 2 h. After 2 h, reaction mixture was cooled to RT and **1** (0.43 g, 2.45 mmol) in 10 mL of acetonitrile (ACN) was added to it and mixture was further stirred at 65 °C for additional 12 h. Reaction mixture was then cooled to RT and concentrated under vacuum. The residue was purified over silica gel column using 1-2 % MeOH-DCM as eluent to obtain the pure product as brown sticky solid (retention factor or RF: 0.4 in 2% MeOH-DCM). Yield: 0.25 g (41%). ^{1}H NMR (500 MHz, CD_3CN): δ 7.57 (brs, 1H), 4.34 (s, 1H), 4.01 (d, 2H, J = 5.2 Hz), 3.94 (s, 2H), 3.04-1.6 (brm, 10H). ^{13}C NMR (125 MHz, CD_3CN): δ 167.0, 75.4, 61.1, 44.0, 28.1.

^{11}B NMR (160 MHz, CD_3CN): δ -1.57, -4.89, -9.53, -11.6, -12.7. HRMS (m/z): Calcd for $C_5H_{16}B_{10}BrNO$ $[M]^-$ 294.1401 Found 294.1630. Calcd for $C_5H_{16}B_{10}BrNO$ $[M+Br]^-$ 373.0596 Found 373.0966.

Synthesis of o-CB-DO3A-t-Bu-ester conjugate (3). In a 100 mL dry round bottom flask, o-CB-2-bromoacetamide conjugate **2** (0.10 g, 0.34 mmol) and DO3A-*t*-Bu-ester (0.17 g, 0.34 mmol) was dissolved in 20 mL of dry ACN. To this mixture *N, N*-diisopropylethylamine, DIPEA (0.11 g, 0.85 mmol) was added and the resultant mixture was stirred at 85 °C for 12 h. After 12 h, reaction mixture was then cooled to RT and concentrated under vacuum. The residue was purified over silica gel column using 2-5 % MeOH-DCM as eluent to obtain the pure product as brown colored sticky solid (RF: 0.4 in 5% MeOH-DCM). Yield: 0.15 g (60%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.31 (t, 1H, J = 6.4 Hz), 4.76 (s, 1H), 3.95 (d, 2H, J = 5.2 Hz), 3.68-1.70 (m, 34H), 1.41 (s, 27H). ^{13}C NMR (100.6 MHz, $CDCl_3$): δ 173.3, 172.9, 172.8, 82.8, 82.7, 76.3, 60.7, 57.0, 56.3, 56.4, 52.8 (broad peak), 49.5 (broad peak), 44.1, 30.3, 28.7, 28.6. ^{11}B NMR (128 MHz, $CDCl_3$): δ -1.99, -5.65, -10.5. HRMS (m/z): Calcd for $C_{31}H_{65}B_{10}N_5O_7$ $[M+Na]^+$ 751.9848. Found 751.9756.

Synthesis of o-CB-DOTA-MA conjugate (4). A mixture of **3** (0.30 g, 0.41 mmol) and formic acid (20 mL, 90%) was stirred for 12 h at 60 °C. Reaction mixture was then concentrated under reduced pressure and dried under high vacuum overnight. The residue was then dissolved in a minimal amount of MeOH, and the crude product was precipitated by adding ether. The precipitate was filtered and washed with ether (3 x 15 mL) and dried in a desiccator under high vacuum to obtain the pure product as white solid. Yield: 0.19 g (82%). MP: 240 °C. ^{1}H NMR (500 MHz, CD_3OD): δ 3.10 (s, 1H), 2.43 (m, 2H), 2.26 (m, 4H), 2.08 (m, 3H), 1.96 (m, 2H), 1.91 (m, 4H), 1.84 (m, 4H), 1.61 (m, 4H), 1.54 (m, 3H), 1.30- 0.00 (m, 10H). ^{13}C NMR (125 MHz, CD_3OD): δ 174.4, 172.3, 169.8, 76.9, 62.5, 57.8, 55.7, 54.5, 52.6, 51.9, 50.1, 49.7, 45.4, 22.0. ^{11}B NMR (160 MHz, CD_3OD): δ -4.14, -7.16, -11.4, -13.1, -14.1. IR (KBr): 3425, 2974, 2854, 2591, 1678, 1634, 1384, 1224, 1086, cm^{-1}. HRMS (m/z): Calcd for $C_{19}H_{41}B_{10}N_5O_7$ $[M-H]^-$ 559.3906. Found 559.3569.

Synthesis of CB-GdDOTA-MA. In a 100 mL round bottom flask, a mixture of **4** (170 mg, 0.30 mmol) and $GdCl_3.6H_2O$ (113 mg, 0.30 mmol) was dissolved in 30 mL H_2O-MeOH mixture

(1:1). The pH of the reaction mixture was adjusted and maintained at 6.5 by adding 1.0 M aqueous NaOH solution. The reaction mixture was stirred for 12 h at RT. After 12 h of stirring, the pH of the reaction mixture was adjusted to 8.5 by adding 1.0 M aqueous NaOH solution and mixture was stirred for additional 2 h and then filtered through 0.2 μm syringe filter. The pH of the solution was adjusted to 7 by addition of 1 M HCl. Next, reaction mixture was concentrated under reduced pressure and crude was subjected to gel-filtration column chromatography on Sephadex® G-25 using 1:1 mixture of H_2O-MeOH. The solvent was removed and crude was lyophilized to obtain **CB-GdDOTA-MA** conjugate as white solid. Yield: 160 mg (73%). MP: 300 °C (decomposed). IR (KBr): 3411, 2988, 2869, 2590, 1610, 1396, 1321, 1083, 939, 722 cm^{-1}. HRMS (m/z): Calcd for $C_{19}H_{38}B_{10}GdN_5O_7$ $[M-H]^-$ 712.2939. Found: 712.2933. ICP-OES analysis: Boron/Gd ratio (10:1).

Synthesis of CB-DyDOTA-MA. In a 100 mL round bottom flask, a mixture of **4** (400 mg, 0.71 mmol) and $DyCl_3.6H_2O$ (269 mg, 0.71 mmol) was dissolved in 30 mL H_2O-MeOH mixture (1:1). The pH of the reaction mixture was adjusted and maintained at 6.5 by adding 1.0 M aqueous NaOH solution. The reaction mixture was stirred for 12 h at RT. After 12 h of stirring, the pH of the reaction mixture was adjusted to 8.5 by adding 1.0 M aqueous NaOH solution and mixture was stirred for additional 2 h and then filtered through 0.2 μm syringe filter. The pH of the solution was adjusted to 7 by addition of 1 M HCl. Next, reaction mixture was concentrated under reduced pressure and crude was subjected to gel-filtration column chromatography on Sephadex® G-25 using 1:1 mixture of H_2O-MeOH. The solvent was removed and crude was lyophilized to obtain **CB-DyDOTA-MA** conjugate as white solid. Yield: 350 mg (68%). HRMS (m/z): Calcd for $C_{19}H_{38}B_{10}DyN_5O_7$ $[M+H]^+$ 720.3128. Found: 720.2835. Calcd for $C_{19}H_{38}B_{10}DyN_5O_7$ $[M+2H]^{2+}$ 360.6600. Found: 360.6457.

HSA Binding studies. The binding of **CB-GdDOTA-MA** with HSA (4.5% w/v = 0.67 mM) in PBS at 7.4 pH and 37 °C was determined using ICP-OES analysis according to the literature reported method.[8] Briefly, 0.1 mM solutions of **CB-GdDOTA-MA** were made in HSA (4.5% w/v = 0.67 mM) in PBS at 7.4 pH. Aliquots (0.4 μL) of these samples were placed in 3 kDa cutoff ultrafiltration units (3 kDa, AMICON, 0.5 μL), incubated at 37 °C for 20 min, and then centrifuged at 14000g for 7 min. The filtrates from these ultrafiltration units were used to determine the unbound conc. of **CB-GdDOTA-MA** by measuring Gd ion conc. of the filtrates

using ICP-OES analysis. The experiments were conducted in a set of 8 ultrafiltration units and the Gd conc. of each filtrate was measured in duplicates. Data presented here is an average of 8 independent ICP-OES analyses of ultra filtrates with the relative uncertainty of <5%.

Determination of hydration number (q) for CB-DyDOTA-MA: Hydration number was determined using a literature reported procedure.[20] Varying concentrations of **CB-DyDOTA-MA** and $DyCl_3.6H_2O$ over the range 10-80 mM were prepared in 80% D_2O-H_2O and the pH of the solutions was adjusted to pH 7. The ^{17}O NMR experiments were performed using a Bruker Ascend 400 MHz NMR instrument at RT with the deuterium signal locked. A graph was plotted between the $\Delta\delta$ and the concentration for both **CB-DyDOTA-MA** and the $DyCl_3.6H_2O$ solutions and slope was obtained (ESI, Fig. S1).

Relaxivity measurement. Solutions of Omniscan and **CB-GdDOTA-MA** were prepared in PBS and 4.5% HSA/PBS at pH 7.4 with a Gd conc. in the range of 0.0625 to 1 mM in 200 µl PCR tubes followed by the MR scan. The measurements were repeated on two or more independently prepared samples to ensure consistency. A buffer matched blank sample (0 mM) was also used in the relaxivity measurements of each sample. To obtain longitudinal relaxation (R1) for samples, inversion recovery spin-echo images were obtained using a sequence with repetition time (TR) /echo time (TE) =3,000 ms / 15 ms, with varied IR (inversion recovery time) from 0.082 to 3.6 s; field of view (FOV) of 60×40 mm; matrix of 128×128; slice thickness (ST) of 1 mm; and number of excitations (NEX) of 2. The final r_1 values were obtained by the linear fitting of relaxation rates against the conc. for each sample.

In vivo MRA Studies. CF1 mice were anesthetized with 1–2% isoflurane in oxygen via a nose cone over the entire imaging period. A respiratory sensor was placed on the abdomen for respiratory monitoring of vital signs using a Physiological Monitoring System (SA Instruments, Inc.; Stony Brook, NY). Body temperature was maintained at 37°C with warm air circulating in the magnet bore. A rapid acquisition with relaxation enhancement (*RARE*) T1-weighted (T1W) imaging sequence was applied to obtain coronal images before and after the 3D contrast enhancement (CE) MRA acquisition. For RARE-T1W sequence: TR / TE = 774 ms / 9 ms, RARE factor = 4, 16 slices, ST = 1 mm with no gap, matrix = 256 × 256, FOV = 80 × 40 mm (coronal), NEX = 4. After the baseline scan had been completed, 200 µl of contrast media was

injected as a bolus via tail vein and dynamic CE-MRA images were obtained continuously for 60 minutes, and then again at 3 and 24 h after injection. Dynamic CE-MRA was acquired using a 3D fast low angle shot (FLASH) sequence as follows: TR / TE = 7.3 ms / 2.3 ms; FOV = 80 × 40 × 20 mm; matrix = 256 x 92 x 32; NEX = 1. Acquisition time was 16.3 seconds. The 3D MRA images were displayed as a MIP (maximum intensity projection). Image analysis and processing were performed with VnmrJ2.1D software (Varian Inc. /Agilent Technologies, 2005). The contrast enhancement ratio (CER) for a tissue was calculated according to the equation:

$$CER = (SI_{post} - SI_{pre}) / SI_{pre} \times 100\%.$$

ICP-OES analysis of tissue samples. To determine the amount of Gd retained in tissues 24 h p.i., mice were euthanized 24 h p.i. and blood, heart, lungs, liver, spleen, kidney, brain, and muscle samples were collected, weighed and immediately frozen at -20 °C. Prior to the ICP-OES analysis, tissue samples were allowed to warm up to room temperature, acid digested, and the gadolinium content of the tissues was determined.

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References and notes

†Electronic Supplementary Information (ESI) available: Copies of HPLC trace, ^{1}H NMR, ^{13}C NMR, ^{11}B NMR, IR and HRMS spectrums of compounds are included.

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Table of content entry

Abstract: The synthesis, relaxivity measurements and *in vivo* assessment of a carborane-GdDOTA-monoamide (**CB-GdDOTA-MA**) amphiphilic conjugate as blood pool contrast agent (BPCA) is reported. This BPCA exhibited excellent binding (87.4%) with human plasma protein (HSA) and showed high relaxivity values (r_1 = 6.8 $mM^{-1}s^{-1}$ at 7 T in PBS).