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Supporting Information

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Supporting Information

for

Gd-DOTA Conjugate of RGD as a Potential Tumor-Targeting MRI Contrast Agent

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1. Materials

Cyclic RGD peptide c(RGDYK) was purchased from Anygen, Korea. 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) was purchased from Mycrocycles, Dallas, TX. 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and *N*-hydroxysulfosuccinimide (sulfo-NHS) were purchased from Fluka. Solvents were dried and purified by standard methods. Semi-preparative reversed-phase HPLC was accomplished on a KNAUER chromatography system with a UV-vis detection probing at 230 nm.

2. Synthesis

2.1. DOTA-RGD: The title compound was prepared according to the known method for the synthesis of DOTA-RGD using cRGDYK.[1] Aqueous solutions of DOTA (96.0 mg, 192.0 μmol, 4.0 mL) and EDC (18.4 mg, 96.0 mmol, 1.0 mL) were mixed in a vial and the pH was adjusted to 5.0 with NaOH (0.1 M). To this mixture on ice bath was added sulfo-NHS (16.8 mg, 76.8 μmol) at 4 ^{o}C, and the pH was further adjusted to 5.5 using NaOH. The reaction mixture was stirred for 30 min at 4 ^{o}C, after which an aqueous solution of c(RGDYK) (12.0 mg, 19.2 μmol, 2.4 mL) was added. After adjusting the pH of the solution to 8.5, the reaction mixture was incubated overnight at 4 °C. The product was purified HPLC using a VYDAC C-18 semi-preparative column (10.0 mm × 250 mm) with the mobile phase starting from 100% solvent A (0.1% TFA in water) (0 – 2 min) to 35% solvent A and 65% solvent B (0.1% TFA in aceto-

nitrile) at 32 min with a flow rate of 3 mL/min. The absorbance was monitored at 230 nm. Retention time for DOTA-RGD conjugate: 14.15 min. MALDI-TOF-MS: m/z 1006.6 for $[M+H]^+$ ($C_{43}H_{68}N_{13}O_{15}$, Calculated MW = 1006.5). The collected DOTA-RGD was lyophilized and stored in a freezer at -20 ^{o}C.

2.2. Gd-DOTA-RGD: $GdCl_3 \cdot 6H_2O$ (175.5 mg, 472 µmol) was dissolved in water (100 mL) in a 500 mL round bottom flask and DOTA-RGD (95.0 mg, 94.4 µmol) in water (100 mL) was added under stirring conditions at RT. The reaction mixture was further stirred for 36 h at RT after which any solid impurities were removed by filtration through Celite. The product was purified by HPLC using a VYDAC C-18 semipreparative column (10.0 mm × 250 mm) with the mobile phase starting from 92% solvent A (0.1% TFA in water) and 8% solvent B (0.1% TFA in acetonitrile) (0 – 8 min) to 77% solvent A and 23% solvent B at 23 min with a flow rate of 3 mL/min. The absorbance was monitored at 230 nm. The retention time for Gd-DOTA-RGD: 9.22 min. MALDI-TOF-MS: *m/z* 1161.5 for $(M+H) - H_2O]^+$ ($C_{43}H_{67}GdN_{13}O_{16}$, calcd M_W = 1178.39). The collected Gd-DOTA-RGD was lyophilized and stored in a freezer at -20 °C.

3. Relaxivity Measurements

T_1 measurements were carried out using an inversion recovery method with a variable inversion time (TI) at 1.5 T (64 MHz). The magnetic resonance (MR) images were acquired at 35 different TI values ranging from 50 to 1750 msec. T_1 relaxation times were obtained from the non-linear least square fit of the signal intensity measured at each TI value. For T_2 measurements, the CPMG (Carr-Purcell-Meiboon-Gill) pulse sequence was adapted for multiple spin-echo measurements. 34 images were acquired with 34 different echo time (TE) values ranging from 10 to 1900 ms. T_2 relaxation times were obtained from the non-linear least squares fit of the mean pixel values for the multiple spin-echo measurements at each echo time. Relaxivities (R_1 and R_2) were then calculated as an inverse of relaxation time per mM. The determined relaxation times (T_1 and T_2) and relaxivities (R_1 and R_2) are finally image-processed to give the relaxation time map and relaxivity map respectively and the results are shown in Table S1.

4. Animal Tumor Model

18-week old H-ras12V transgenic mice bearing hepatocellular carcinoma (35 g) were employed for the present studies. Each animal was anaesthetized with an intra-

muscular injection of a mixture of xylazine (50 µL; Rompun®: 20 mg/mL) and ketamine (10 µL; Ketalar®: 50 mg/mL) before injecting the CAs. For MR imaging in the targeting experiment, Gd-DOTA-RGD was injected into the tail vein of the animal with a dose of 1.43 mmol/kg. For the blocking experiment, c(RGDYK) (1.43 mmol/kg) was injected first and followed by the same dose of Gd-DOTA-RGD after 30 min. For MR imaging with Omniscan®, same dose as that of Gd-DOTA-RGD was injected under identical conditions. Since the Institutional Animal Care and Use Committee of Kyungpook National University is expected to be formed by the end of 2008, no rules are currently in place for animal experiment in the institution.

5. In vivo Animal Imaging

MR images of anesthetized mouse before and after injections of this CAs taken at the interval of every 10 min upto 270 min were obtained with a 1.5 T scanner (GE Signa Advantage, GE Medical system, USA) and extremity coil. The animals were placed in the magnet in a supine position with the heads firmly fixed. After each measurement, the animals were revived from anesthesia and placed in their cages with free access to food and water. During MRI measurements, the animals were maintained at 37.0 °C using a warm water blanket. The imaging parameters for SE (spin echo) were as follows: repetition time (TR) = 500 ms; echo time (TE) = 12 ms; 80 mm field of view (FOV); 60 mm phase FOV; 192×128 matrix size; 15 axial slices; 2 mm slice thickness; slice gap of 0 mm; number of acquisition (NEX) = 4.

6. Histological Analysis

Random sections of 5-µm thickness were taken from the tumor nodules and stained with antibodies CD31 (Abcam? , Cambridge, UK) and Envision HRP (DaKo North America Inc, CA, USA). At least approximately 47 random fields were photographed and analyzed from each slide using an Olympus microscope (BX54), equipped with a digital camera (Figure S6). A computer-based quantitative analysis of immuno-histochemical staining was performed. Briefly, the CD31-positive areas were extracted from the photographs using MATLAB software (The Mathworks™, MI, USA). Vascular density was measured as the area of CD31-positive cells per area unit. All data are presented as mean ± SD, unless specified. The means of two groups were compared using the two-tailed Student *t*-test. The Gd-content of the remaining tumor was obtained from ICP-AES analysis (Figure S7).

7. MR signal analysis

The anatomical locations with enhanced contrast were identified with respect to hepatocellular carcinoma of the liver on post-contrast MR images. For SNR and CNR calculations, signal intensities in specific regions of interest (ROI) of 20 – 40 mm^2 were measured using Advantage Window software (GE Medical, USA). These data were analyzed with MATLAB 7.1.0.246 (R14) Service Pack 3 (The Mathworks^{TM}, MI, USA) to get the color-grading shown in Fig. 1.

The signal-to-noise ratio (SNR) is defined as the ratio of mean signal intensity the anatomic ROI to that of the background noise. The contrast-to-noise ratio (CNR) is defined as the difference in SNR between adjacent anatomic structures.

$CNR = SNR_{after} - SNR_{before}$

The normalized signal was calculated as follows:

$$\text{Normalized signal} = \frac{CNR}{CNR_{\max}} \times 100$$

8. Cytotoxicity Assay

14D Chick cornea stroma primary cells (p1) were used. These cell lines were obtained from Department of Biology, College of Natural Sciences, Kyungpook National University. The cells were grown in 100 cm^2 plastic culture dishes (Corning® Culture Dish) in 10 mL of medium at 37 ^{o}C and in a humidified 5% CO_2 atmosphere. Cells were maintained in F-12-medium (Gibco®) supplemented with heat-inactivated 10% FCS, 1% chicken serum, 5 mg/mL insulin, 10 ng/mL human recombinant EGF, 100 IU/mL penicillin, 100 mg/mL streptomycin and 200 mg/mL gentamicin (all purchased from Gibco®). The medium was replaced every 2 days, and cells were split into 96-well plate (6 x 10^4 cells/well/200 µL). Various concentrations (0.2 – 500 µM) of Gd complexes were added into the culture serum free media and incubated for 24 h. Cell viability/toxicity assessment was performed by using Cell Counting Kit (CCK-8), which was purchased from Dojindo Laboratory, Japan. 10 µL of CC8-kit solution was directly added to each well and the plate was incubated at 37 °C for 3 h. The O.D. was read at 490 nm using an ELISA (Molecular Device, USA Bio-rad 550 Reader) to determine the cell viability/toxicity in each well.

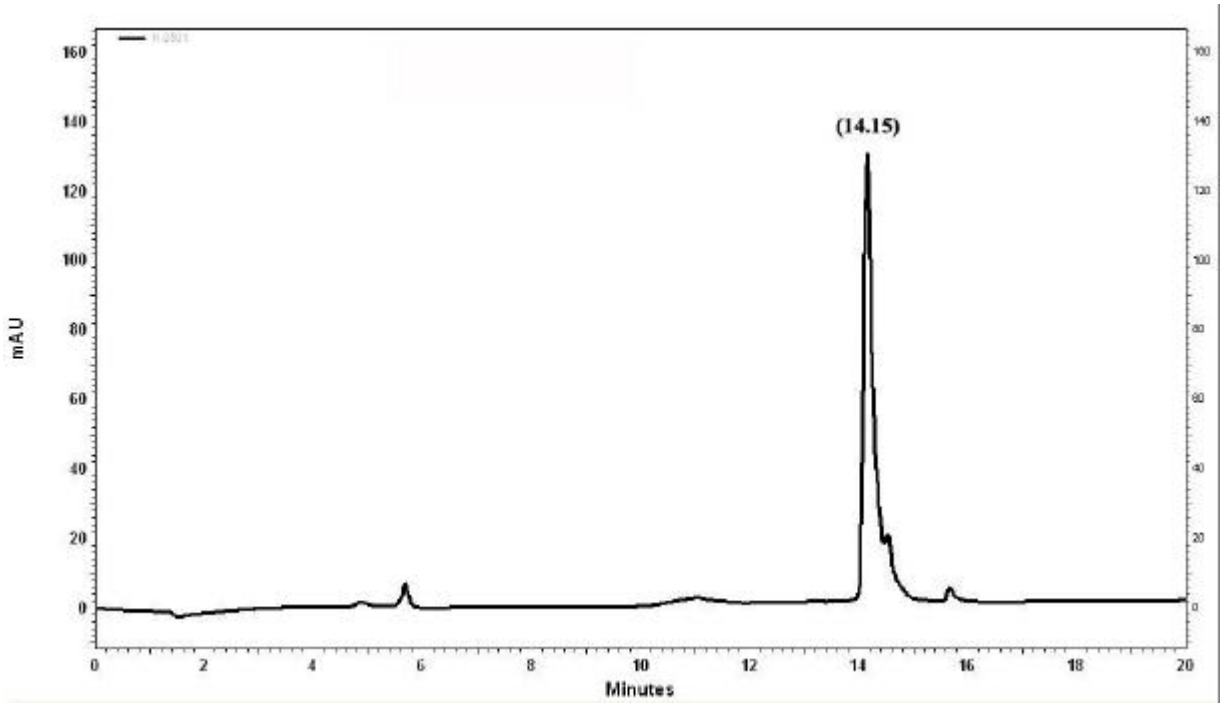

Figure S1. HPLC of DOTA-RGD.

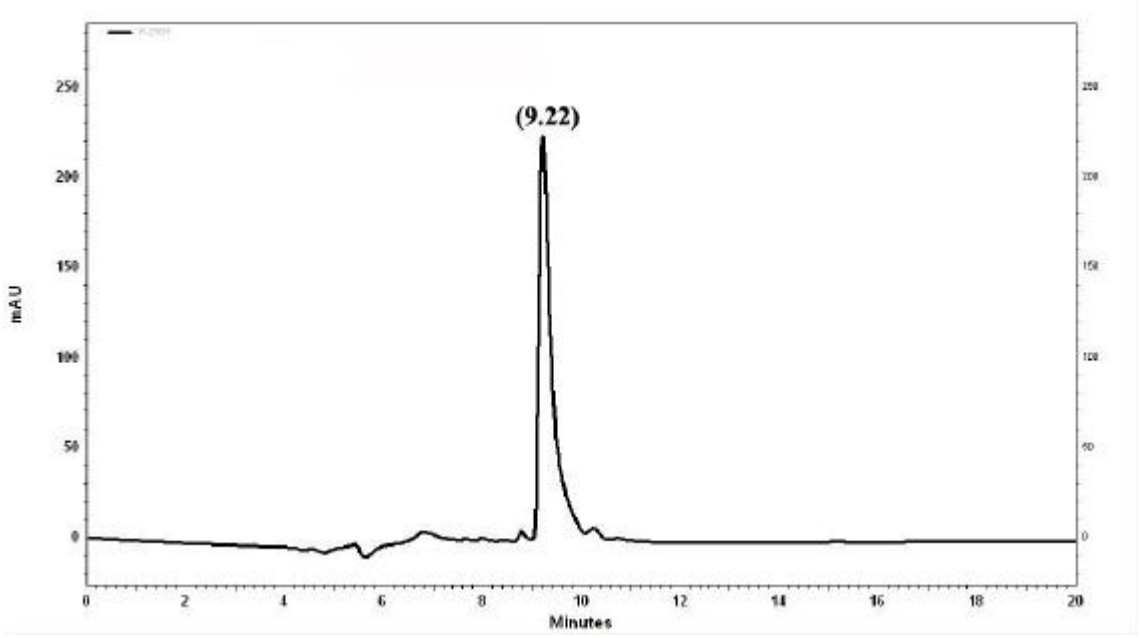

Figure S2. HPLC of Gd-DOTA-RGD.

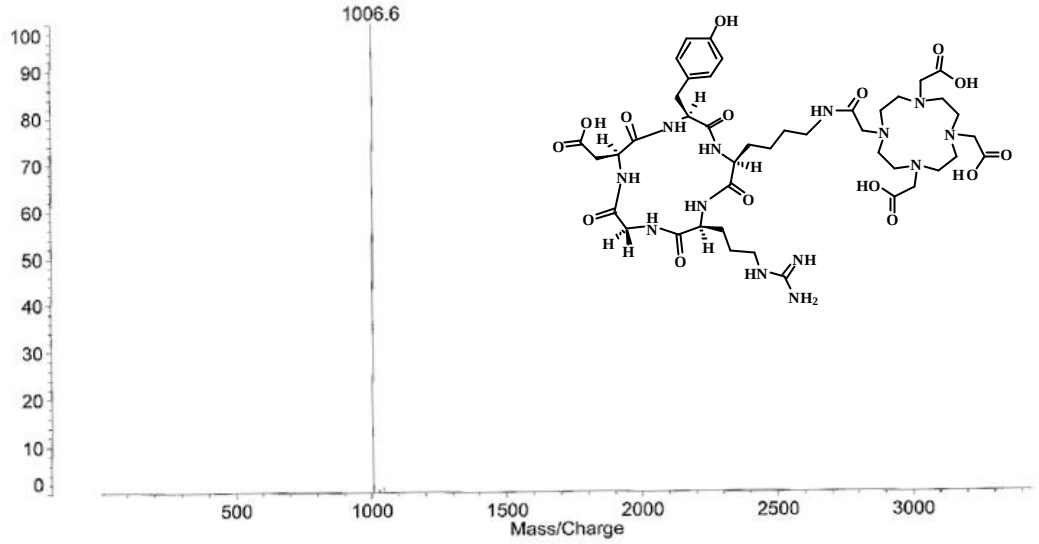

Figure S3. The Maldi-TOF mass spectrum of DOTA-RGD.

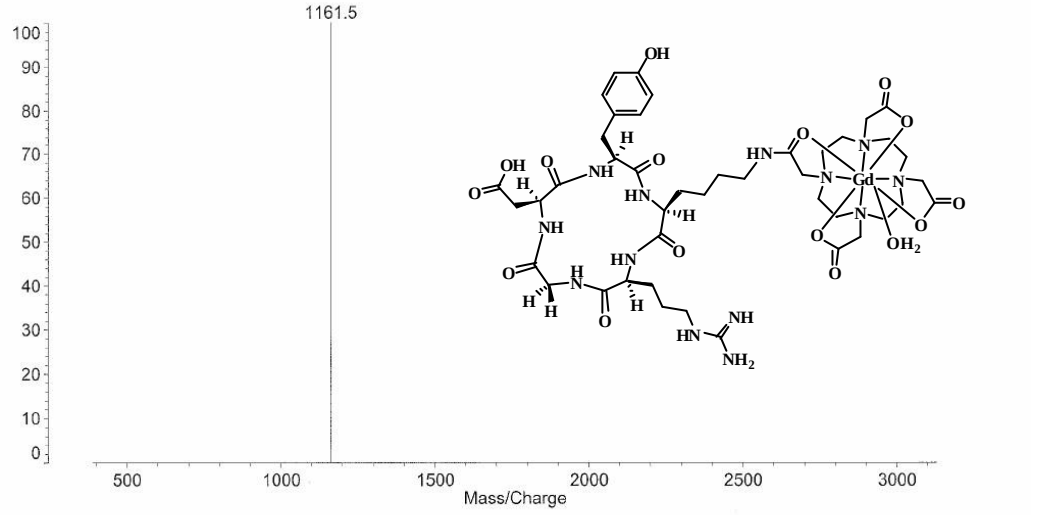

Figure S4. The Maldi-TOF mass spectrum of Gd-DOTA-RGD.

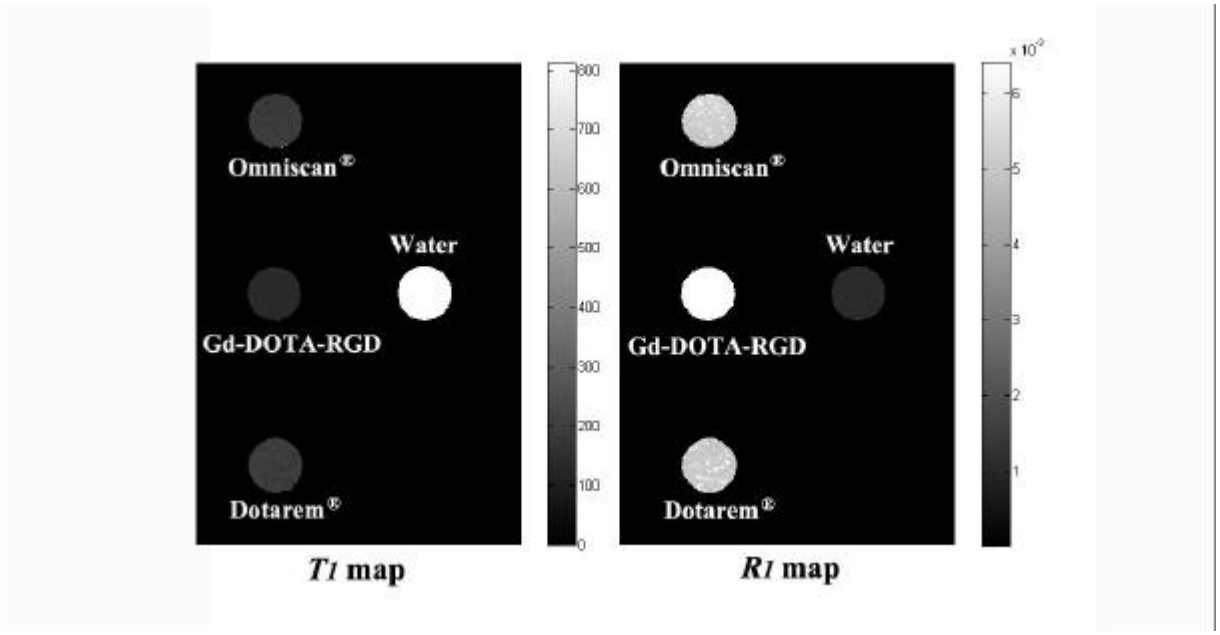

Figure S5. T_1 and R_1 maps of Gd-DOTA-RGD, Omniscan®, and Dotarem®.

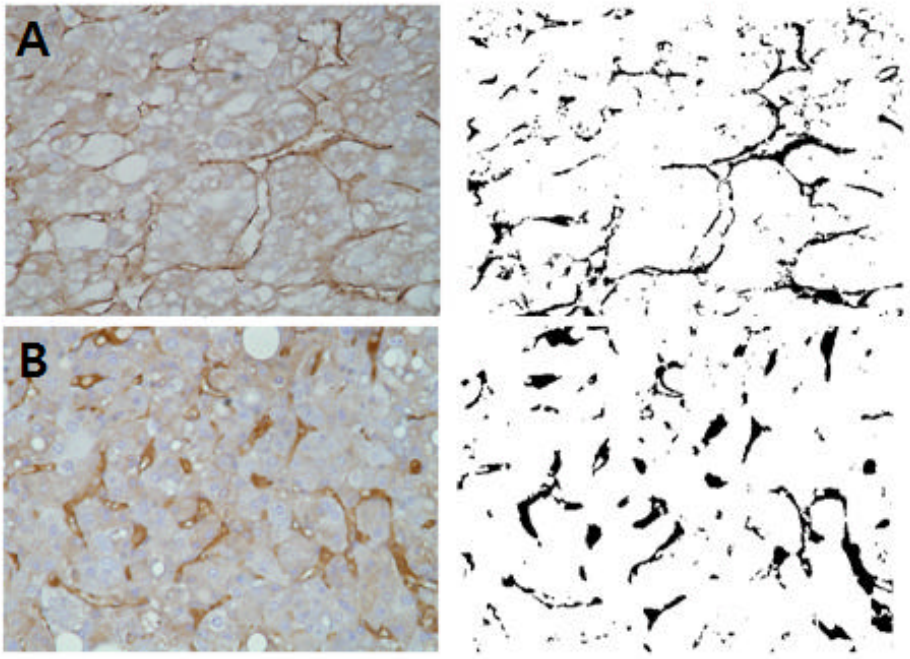

Figure S6. CD-31 immunohistochemical staining and segmented images of vasculatures from two different mH-Ras mouse with hepatocyte cellular carcinoma (HCC): A-targeting and B-blocking. Image segmentation was performed to calculate vascular area quantitatively.

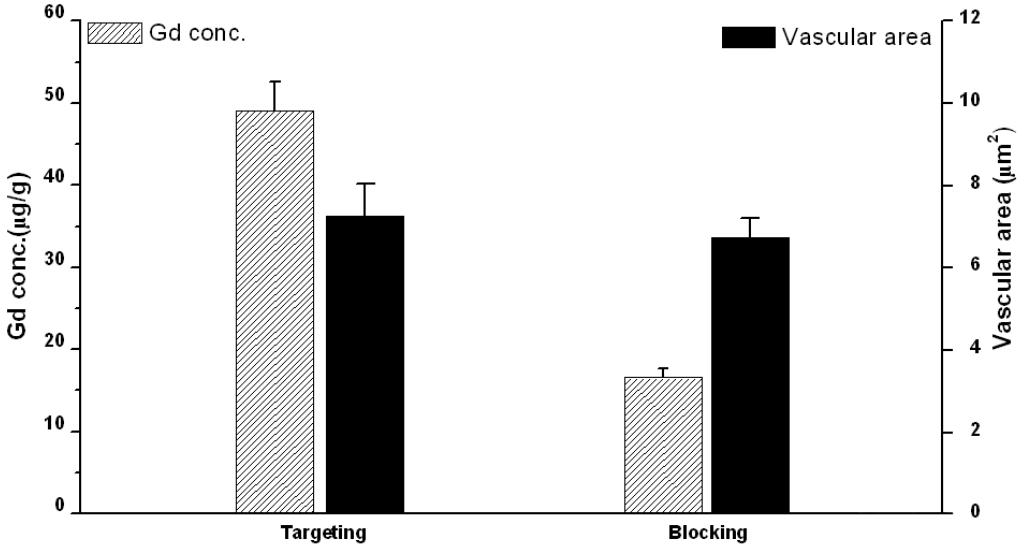

Figure S7. Gadolinium concentrations and vascular areas of the tumor.

Table S1. Relaxation tmes (T_1, T_2) and Relaxivities (R_1, R_2) for Gd-DOTA-RGD, Omniscan®, Dotarem®, and water with the ±SDs.

Sample	T_1 [ms]	R_1 [$mM^{-1}s^{-1}$]	T_2 [ms]	R_2 [$mM^{-1}s^{-1}$]
Gd-DOTA-RGD	135.45 ± 3.71	7.4 ± 0.20	252.62 ± 15.04	4.0 ± 0.24
Omniscan®	189.54 ± 10.26	5.3 ± 0.33	371.36 ± 20.61	2.7 ± 0.15
Dotarem®	192.81 ± 11.85	5.2 ± 0.36	330.30 ± 15.94	3.0 ± 0.15
Water	908.86 ± 26.79	1.1 ± 0.03	878.67 ± 35.38	1.1 ± 0.05

References

1. X.Chen, R. Park, M. Tohme, A. H. Shahinian, J. R. Bading, P. S. Conti, *Bioconjugate Chem.,* **2004**, *15*, 41.

2. M.-I. Huh, Y.-M. Lee, S.-K. Seo, B.-S. Kang, Y. Chang, Y.-S. Lee, M. E. Fini, S.-S. Kang, J.-C. Jung, *J. Cell Biochem.* **2007**, *101,* 1222.