EXPERIMENTAL PROCEDURES

Protected amino acids for peptide synthesis were purchased from Novabiochem (Burlington, MA, USA). N,N-Diisopropylethylamine (DIPEA) was bought from MP Biomedical LLC (Santa Ana, CA, USA). O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluorophosphate (HBTU) was purchased from Anaspec Inc (Fremont, CA, USA). 5-Hexynoic acid was purchased from Sigma-Aldrich (St. Louis, MO, USA). Ascorbic acid (99%) was obtained from Acros organics (Thermo Fisher, Waltham, MA, USA). PC3 and LNCaP cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in RPMI medium (Thermo Fisher) supplemented with 10% fetal bovine serum (Gibco, Gaithersberg, MD, USA), 100 U/mL penicillin, and 0.1 mg/mL streptomycin (Thermo Fisher) at 37 °C in 5% CO2. All other reagents were obtained from Thermo Fisher and used without any purification unless mentioned otherwise.

Animal tumor models

Male athymic nude mice were purchased from the Case Comprehensive Cancer Center (Cleveland, OH, USA), and cared for in the Animal Core Facility of Case Western Reserve University. All experiments were conducted in accordance to an animal protocol approved by the CWRU Institutional Animal Care and Use Committee. Upon anesthetization, tumor cells were injected into the flanks ($4.0*10^6$ cells/mL) in a PBS/matrigel mixture (Corning, Bioscience, Corning, NY) to induce tumors. Tumor growth was monitored via caliper measurements until tumors reached a diameter ranging from 5-8 mm.

Synthesis of alkynyl-ZD2

ZD2 peptide was synthesized using solid phase chemistry as previously described[10, 14]. The mixture of 5-hexynoic acid (3 eq.), HBTU (3 eq.), DIPEA (3 eq.) in 10 mL dry DMF was added to the resin at the end of peptide synthesis (1.5 mmol peptide) and was shaken for 6h. The resin then was filtered and washed with DMF (10 ml × 3) and DCM (10 ml × 3). Alkynyl-ZD2 was subsequently cleaved from the resin for 3 hours using a cocktail of TFA:H2O:TIBS (96.5:2.5:1). Alkynyl-ZD2 was precipitated in cold ethyl ether, centrifuged and lyophilized. The product was characterized by MALDI-TOF mass spectrometry, m/z (M+1): 843.57; 843.413. (calc.)

Synthesis of ZD2-N3-Gd(HP-DO3A)

The azido-containing macrocyclic Gd(III) chelate, N3-Gd(HP-DO3A) was prepared according to a published method[17]. Alkynyl-ZD2 (0.40 mmol, 0.34 g) and N3-Gd(HP-DO3A) (0.60 mmol, 0.36 g) were dissolved in 25 mL deionized water. CuSO4 (1.6 ml, 0.05 N), ascorbic acid (5 ml, 0.05 N in water) were added, the pH of solvent was adjusted to 8~9 by 0.1 N NaOH, and the mixture was stirred at room temperature for 24 h. The solvent was removed to obtain the crude product, which was purified by FLASH collector (Biotage® HP-Sphere C18 25 µm, Charlotte, NC, USA). The gradient elution used was 100% water for 42 ml, 0-20% acetonitrile in water for 60 ml, 20-50% acetonitrile in water for 30 ml and 50-100% acetonitrile in water for 30 ml. The product fraction was collected and the solvent was removed to give an off-white solid product. The final product was characterized by MALDI-TOF mass spectrometry, m/z (M+H+) 1443.73; 1443.54 (calc.).

HPLC Characterization

The Gd-compound was separated by high performance liquid chromatography (HPLC) using Agilent 1100 series system on a C18 column (Ultrasphere ODS, 5 µm, 4.6x250 mm), with a linear gradient of acetonitrile (0-30%) in water and 0.1% TFA at flow rate of 1 ml/min for 20 min. Elution of the Gd-compound was detected at 210 nm at room temperature.

Relaxivity Measurement

Solutions of ZD2-N3-Gd(HP-DO3A) were prepared at concentrations of 0.125, 0.25, 0.5, 1, and 2.5 mM in PBS buffer (pH 7.4). The T1 and T2 of the ZD2-N3-Gd(HP-DO3A) solutions at 1.4 T was measured using a Bruker Minispec Relaxometer (60 Hz, 37°C). The T1 values of the solutions were also measured using 7 T Bruker Biospec small animal scanner (Bruker Biospin Co., Billerica, MA) with a built-in EPI sequence with the following parameters: TR = 10000 ms, TE = 26 ms, FOV = 7.00 cm x 7.00 cm, slice thickness = 2 mm, Nav = 1, matrix = 64 x 128, acquisition time = 10 seconds. T2 values at 7 T were determined using a multi-slice multi-echo sequence with the following parameters: TR = 3305.7 ms, TE = 100 ms, Refocussing FA = 180°, FOV = 7.00 cm x 7.00 cm, slice thickness = 2 mm, Nav = 1, matrix = 128 x 128, acquisition time = 5 min 17 seconds. The r1 and r2 relaxivities were calculated from the slopes of 1/T1 and 1/T2 vs. Gd concentration plots, respectively.

Contrast Enhanced MRI

Contrast enhanced MRI experiments were performed on a 7 T Bruker small animal MRI scanner. Mice bearing PC3 or LNCaP prostate tumor xenografts were anaesthetized with 2% isoflurane. A 30-gauge needle connected with a tubing (1.6 m) was fixed into the tail vein. Mice were then placed in the magnet and kept under anesthesia with 1.5% isoflurane. Body temperature was maintained at 37 °C by blowing warm air into the magnet. MR images were taken using a Bruker Biospec

7 T MRI scanner with a volume radiofrequency (RF) coil. After the pre-injection baseline MR images had been taken, the contrast agent was injected at a dose of 0.1 mmol Gd/kg, and was subsequently flushed with 100 μL of saline. 3D FLASH coronal images were acquired before and at 2, 10, 20, 30 min post injection using the following parameters: TR = 15 ms, TE = 2.8 ms, FA = 15°, FOV = 10.00 cm x 3.00 cm, slice thickness = 35 mm, Nav = 1, matrix = 128 x 512, resolution = 0.0127 x 0.273 cm/pixel, acquisition time = 61 s. T1 weighted-2D axial images were acquired immediately after each 3D FLASH acquisition using multi-slice multi-echo sequence: TR = 500 ms, TE = 8.1 ms, FA = 90°, FOV = 3.50 cm x 3.50 cm, slice thickness = 1.20 mm, slice number = 16, Nav = 2, matrix = 128 x 128, acquisition time = 128 seconds. Each experimental group contained 4 mice.

MRI data analysis

Image analysis was conducted using the Bruker Biospec Topspin software and OsiriX software. Regions of interest were defined in the tumor, kidneys, aorta and muscle and used to calculate the contrast-to-noise ratio (CNR) using the following equation: $CNR = (S_T-S_M)/\sigma_N$, where S_T and S_M are the signal intensity from the ROIs of tumor, kidney, aorta and muscle regions, respectively, and σ_N is the standard deviation of noise estimated from background air.