Gd-AAZTA-MADEC, an improved blood pool agent for DCE-MRI studies on mice on 1 T scanners

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Running title

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Full paper

ABSTRACT

A novel MRI blood-pool contrast agent (Gd-AAZTA-MADEC) has been compared with established blood pool agents for tumor contrast enhanced images and angiography. Synthesis, relaxometric properties, albumin binding affinity and pharmacokinetic profiles are reported. For *in vivo* studies, angiographic images and tumor contrast enhanced images were acquired on mice with benchtop 1T-MRI scanners and compared with MS-325, B22956/1 and B25716/1. The design of this contrast agent involved the elongation of the spacer between the targeting deoxycholic acid moiety and the Gd-AAZTA imaging reporting unit that drastically changed either the binding affinity to albumin (K_A(HSA) = $8.3x10^5$ M^{-1}) and the hydration state of the Gd ion (q=2) in comparison to the recently reported B25716/1. The very markedly high binding affinity towards mouse and human serum albumins resulted in peculiar pharmacokinetics and relaxometric properties. The NMRD profiles clearly indicated that maximum efficiency is attainable at magnetic field strength of 1T. *In vivo* studies showed high enhancement of the vasculature and a prolonged accumulation inside tumor. The herein reported pre-clinical imaging studies show that a great benefit arises from the combination of a benchtop MRI scanner operating at 1T and the albumin-binding Gd-AAZTA-MADEC complex, for pursuing enhanced angiography and improved characterization of tumor vascular microenvironment.

Keywords: Magnetic resonance imaging (MRI); Gadolinium contrast agent; blood pool imaging; angiography; human serum albumin; DCE-MRI

INTRODUCTION

The advent of Molecular Imaging era has witnessed the introduction of Magnetic Resonance Imaging (MRI) pre-clinical scanners operating at lower magnetic field strength than the complex (and expensive) high field systems that are found in the specialized MR spectroscopy/imaging labs. The need for making MRI as one of the complementary imaging techniques for multi-modal studies has stimulated manufactory companies to consider the possibility of offering scanners based on permanent or electro-magnets and characterized by a user-friendly images acquisition procedure. It is expected that the availability of this new generation of MRI scanners will be a key-components for the spreading of *in vivo* Molecular Imaging facilities in biological departments together with compact PET/SPECT and Optical Imaging instruments.

In this context it appears likely that MRI scanners will be exploited in pre-clinical cancer research for evaluating novel cancer treatment approaches that require imaging methods able to accurately detect and characterize individual tumors. The last 25 years of MRI clinic studies has clearly demonstrated the important results that may be obtained by the use of paramagnetic Gd-complexes. Gd-based complexes have been extensively used in order to improve the contrast efficiency of the MRI modality, thus providing earlier tumor detection, staging and assessment of therapeutic response [1].

Two key applications of Gd-based contrast agents (CAs) appear of general use in the MRI study of tumor murine models, namely the visualization of the blood vessel network and the assessment of vascular permeability [2]. The best results for both applications have been obtained by using Gd(III) complexes able to reversibly bind to serum albumin [3-6]. The formation of supramolecular adducts between the paramagnetic complex and the serum protein has beneficial effects both on the attainable relaxation enhancement (particularly high at 0.5-1.5 T) and on the *in vivo* distribution properties. In fact, macromolecular Gd complexes have longer blood pool retention time while their reduced tumbling time (rotational correlation time, τ_R) increases their efficiency to relax water protons, in particular at magnetic field around 1T [7, 8]. Moreover, macromolecular CAs can preferentially accumulate in tumor tissue due to the hyperpermeability of tumor vasculature, resulting in effective tumor enhancement for precise cancer detection and delineation [9]. The longer circulation time of macromolecular CAs can also be exploited for characterizing tumor microvasculature with dynamic contrast enhanced (DCE) MRI technique, which allows to assess tumor vessel permeability/perfusion as well as to monitor non-invasively tumor response to

anticancer treatment [10, 11]. In addition, it is often recognized that CAs bigger in size are more efficient reporters on tumor permeability than small size CAs, because of the slower wash-in/wash-out characteristics [12, 13]. Furthermore, the efficiency of a Gd complex to enhance the relaxation rate of water protons depends on the structure of the complex, the strength of the applied magnetic field and on the albumin binding affinity that need to be addressed, simultaneously, in order to improve its contrast efficiency [14-19].

In this study, we introduce a novel Gd-based CA as a blood pool and tumor vascular permeability agent and its properties are compared with those ones of the clinically approved MS-325 (Vasovist or Ablavar) [20], with B22956/1 [21], a blood-pool CA tested in Phase I trials and with B25716/1 [22].

MATERIALS AND METHODS

Chemistry

Commercially available reagents and solvents were purchased from Sigma-Aldrich or Alfa-Aesar and used without further purification. B22956/1 [23], B25716/1 and MS-325 were kindly provided by Bracco Imaging (Milan, Italy) and their chemical structure are shown in Fig. 1a-c. The protected bifunctional chelating agent 1 was prepared according to ref. [24] while methyl 3-aminodeoxycholate 2 was synthesized following the procedure reported in ref. [25]. Reactions were monitored by TLC on Merck 60F254 (0.25 mm) plates. Spot detection was carried out by staining with an alkaline $KMnO_4$ solution or with the Dragendorff reagent. NMR spectra were recorded at 298K on a Jeol Eclipse ECP300 spectrometer operating at 7.05 T; chemical shifts (δ) are given in ppm, coupling constants (J) in Hz. ESI mass spectra were recorded on ThermoFinnigan LCQ Deca XP-Plus and melting points (uncorrected) with a Stuart Scientific SMP3 apparatus. Human serum albumin and mouse serum albumin were purchased from Sigma-Aldrich.

Synthesis

The synthetic procedure leading to Gd-AAZTA-MADEC is reported in Scheme 1.

Synthesis of conjugate 3

Compound **1** [18] (14.0 g, 18.9 mmol) and methyl 3-aminodeoxycholate [19] (**2**, 8.50 g, 21.0 mmol) were dissolved in dichloromethane (100 mL). *N,N'*-Dicyclohexylcarbodiimide (4.9 g, 23.7 mmol) and 4-dimethylaminopyridine (0.46 g, 3.8 mmol) were added to the solution and stirred at room temperature overnight. The white solid precipitate was removed by filtration on a Buchner funnel and the filtrate evaporated under reduced pressure. The residue was submitted to chromatographic purification, obtaining the desired product as a white solid (13.6 g, 64%). M.p. 70°C. ^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 5.73 (d, J = 7.4 Hz, 1H), 4.11 (m, 1H), 3.92 (m, 1H), 3.60 (s, 3H), 3.57 (s, 4H), 3.17 (s, 4H), 2.93 (d, J = 14.1 Hz, 2H), 2.76-2.54 (m, 4H), 2.58 (d, J = 14.1 Hz, 2H), 2.36-0.95 (m, 43H), 1.385 (s, 18H), 1.382 (s, 18H), 0.91 (d, J = 6.4 Hz, 3H), 0.90 (s, 3H), 0.62 (s, 3H); ^{13}C-NMR ($CDCl_3$, 75.4 MHz): δ = 174.7 [C], 172.9 [C], 172.3 [C], 170.9 [C], 80.7 [C], 80.2 [C], 73.0 [CH], 65.4 [CH_2], 63.1 [C], 62.6 [CH_2], 59.3 [CH_2], 52.0 [CH_2], 51.5 [CH_3], 48.9 [CH], 48.3 [CH], 47.3 [CH], 46.5 [C], 45.1 [CH_2], 38.1 [CH], 37.6 [CH_2], 37.1 [CH_2], 35.9 [CH], 35.1 [CH], 34.7 [CH_2], 34.0 [CH_2], 33.0 [CH], 31.1 [CH_2], 31.1 [CH_2], 31.0 [CH_2], 30.6 [CH_2], 30.5 [CH_2], 29.7 [CH_2], 29.6 [CH_2], 29.4 [CH_2], 29.3 [CH_2], 29.0 [CH_2], 28.3 [CH_3], 28.2 [CH_3], 27.5 [CH_2], 26.7 [CH_2], 25.9 [2xCH_2], 25.7 [CH_2], 25.0 [CH_2], 24.8 [CH_2], 23.9 [CH/CH_3], 23.7 [CH_2], 22.1 [CH_2], 17.4 [CH_3], 12.8 [CH_2]; MS (ESI+): m/z calculated for $C_{64}H_{113}N_4O_{12}$ $[M+H]^+$: 1129.84; found: 1129.93.

Synthesis of ligand AAZTA-MADEC

Conjugate **3** (13.5 g, 12.0 mmol) was dissolved in a mixture of 2-propanol (100 mL) and a solution of sodium hydroxide (1.23 g) in deionized water (370 mL). The reaction mixture was stired at room temperature for 48h, then evaporated under reduce pressure. The residue was redissolved in dichloromethane (20 mL) and trifluoroacetic acid (25 mL) was added, stirring the resulting mixture at room temperature overnight. Volatiles were evaporated and the residue triturated and washed with acetone (50 mL), obtaining ligand **AAZTA-MADEC** (10.5 g). ^{1}H-NMR (DMSO-d_6, 300 MHz): δ = 7.35 (d, J = 6.7 Hz, 1H), 3.97 (m, 1H), 3.80 (m, 1H), 3.63 (s, 4H), 3.53 (s, 4H), 3.11-2.93 (m, 4H), 3.03 (d, J = 14.7 Hz, 2H), 2.98 (d, J = 14.7 Hz, 2H), 2.29-0.87 (m, 42H), 2.10 (t, J = 7.2 Hz, 2H), 0.94 (d, J = 6.4 Hz, 3H), 0.91 (s, 3H), 0.63 (s, 3H). ^{13}C-NMR (DMSO-d_6, 75.4 MHz): δ = 175.2 [C], 175.1 [C], 172.3 [C], 171.0 [C], 71.9 [CH], 63.1 [C], 61.9 [CH_2], 59.8 [CH_2], 55.7 [CH_2], 52.3 [CH_2], 48.1 [CH], 47.0 [CH], 46.8 [C], 45.1 [CH], 37.2 [CH], 36.3 [C], 36.2 [CH], 36.0 [CH_2], 35.4 [CH], 34.8 [CH_2], 33.8 [CH_2], 33.1 [CH], 31.6 [CH_2], 31.4 [CH_2], 31.13 [CH_2], 31.06 [CH_2], 30.3 [CH_2], 29.5 [CH_2], 29.4 [2xCH_2], 29.3 [CH_2], 29.2 [CH_2], 27.6 [CH_2], 27.1 [CH_2], 26.3 [CH_2], 26.0 [CH_2], 25.9 [CH_2], 25.1 [CH_2], 24.9 [CH_2], 24.0 [CH_2], 23.8 [CH_3], 23.0 [CH_2], 17.6 [CH_3], 13.0 [CH_3]; MS (ESI-): m/z calcd for $C_{47}H_{77}N_4O_{12}$ $[M-H^+]^-$: 889.55; found: 889.65.

Preparation of Gd-AAZTA-MADEC

Ligand **AAZTA-MADEC** (9.0 mmol) was suspended in deionized water (30 mL) and freshly prepared $Gd(OH)_3$ (9.0 mmol) was added. The opalescent suspension was stirred at 80°C until complete solution was obtained (~32h), periodically checking the presence of free Gd with Xylenol Orange. The solution was then filtered on 0.25 μm filters and evaporated under reduced pressure. The crude product was redissolved in deionized water (10 mL) and precipitated with acetone (50 mL) (3 times), then the solid was dried under vacuum to constant weight to obtain **Gd-AAZTA-MADEC** as a white powder (7.0 g).

Water proton relaxivity measurements

The longitudinal water proton relaxation rates were measured by using a Stelar Spinmaster (Mede, Pavia, Italy) spectrometer operating at 0.47 T by the standard inversion-recovery technique (16 experiments, 2 scans). A typical 90° pulse width was 3.5 ms and the reproducibility of the T1 data was ±0.5%. The temperature was controlled with a Stelar VTC-91 air-flow heater equipped with a copper/constantan thermocouple (uncertainty ±0.1°C). The proton 1/T1 NMRD profiles were measured over a continuum of magnetic field strength from 0.00024 to 0.47 T (corresponding to a 0.01–20 MHz proton Larmor frequency) on a Stelar field-cycling relaxometer. The relaxometer operates under complete computer control with an absolute uncertainty in 1/T1 of ±1%. Data points from 0.47 (20 MHz) to 1.7 T (70 MHz) were collected on a Stelar Spinmaster spectrometer operating at variable fields.

Binding to Human Serum Albumin (HSA)

Binding parameters (the affinity constant K_A, the number of equivalent and independent binding sites n) and the relaxivity of supramolecular adduct r_1^b were determined using the proton relaxation enhancement (PRE) method [26]. The method is based on the titration of a fixed concentration of Gd-complex with increasing concentrations of macromolecule that results in an increase of relaxation rate. The fitting of the obtained curve affords the value of nK_A and r_1^b.

Animals

Male BALB/c mice and male C57BL/6 were obtained from Charles River Laboratories (Calco, Italy). Studies were approved by the local ethics committee of our University and carried out in

accordance with the EU guidelines. All animals were maintained under specific pathogen-free conditions inside the animal facility and received standard rodent chow and had free access to tap water.

Pharmacokinetic Study

Plasma pharmacokinetic was assessed on male BALB/c mice (18-20 g). A group of six mice were used for each contrast agent. The contrast agent was injected at a dose of 0.05 mmol Gd/kg body weight (b.w.). Blood samples (50 µL) were collected from the tail vein before injection and at 5, 30, 60, 120, 360 min after injection for a total of six time points. The blood samples were centrifuged at 1000 rpm at 4°C for 10 min to obtain plasma. The plasma was diluted with sterile water (Baxter) and the Gd content was determined by inductively coupled plasma mass spectrometry (ICP-MS) (Element-2; Thermo-Finnigan, Rodano (MI), Italy). Sample digestion was performed with 1 mL of concentrated HNO_3 (70%) under microwave heating (Milestone MicroSYNTH Microwave labstation). A two-compartment pharmacokinetic model was used to analyze the data and to calculate the pharmacokinetic parameters such as distribution and elimination half-lives ($T_{d1/2}$, $T_{e1/2}$) from the percentages of the initial blood concentration C_0 with GraphPad Prism software (GraphPad, San Diego, CA, USA).

Magnetic Resonance Angiography

A group of six male BALB/c mice weighing approximately 20-24 grams were used in contrast enhanced MR blood pool imaging for each agent. Mice were anesthetized by injecting a mixture of tiletamine/zolazepam (Zoletil 100; Virbac, Milan, Italy) 20 mg/kg and xylazine (Rompun; Bayer, Milan, Italy) 5 mg/kg and placed supine in a solenoid Tx/Rx coil with an inner diameter of 3.5 cm. The contrast agents were injected via a tail vein at a dose of 0.05 mmol Gd/kg.

MR images were acquired before and at 2, 5, 15, 30, 45 and 60 min post-injection of the contrast agents on a 1T MRI Bruker Icon^{TM} system (Bruker BioSpin MRI, Ettlingen, Germany). A 35 mm Tx/Rx mouse solenoid whole body coil was used for both RF excitation and reception of MR signal. A 3D GRE fast low angle shot (FLASH) pulse sequence (TR 10 ms; TE 4.1 ms; flip angle 40°; FOV 80x40x40 mm; MTX 192x156x156; NEX 2; temporal resolution 3 min 4 s per image) with an isotropic spatial resolution of 416 µm was used for image acquisition. Three dimensional

maximum intensity projection (MIP) images were reconstructed using the ImageJ program (http://rsb.info.nih.gov/ij/) by subtracting pre-contrast to post-contrast images.

DCE-MRI in mice tumor model

B16F10 murine melanoma cells were cultured in 75-cm^2 flasks in a humidified incubator at 37 °C and at CO_2/air (5:95 v/v) in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 100U/mL penicillin and 100 mg/mL streptomycin. The cells were allowed to grow to about 90% confluence and then resuspended in DMEM medium. The cell number was adjusted to 10^6 cells/mL containing approximately $2.0x10^5$ cells in 0.2 mL and implanted subcutaneously in the flanks of C57BL/6 mice (six mice for each investigated contrast agent). Tumor size reached 0.4–0.6 cm in diameter 14-20 days after cell implantation.

Magnetic resonance images were acquired on anesthetized mice with an Aspect M2 MRI System (Aspect Magnet Technologies Ltd., Netanya, Israel) working at 1 Tesla. Mice were anesthetized by injecting a mixture of tiletamine/zolazepam (Zoletil 100; Virbac, Milan, Italy) 20 mg/kg and xylazine (Rompun; Bayer, Milan, Italy) 5 mg/kg and placed supine in a solenoid Tx/Rx coil with an inner diameter of 3.5 cm. Breath rate was monitored throughout *in vivo* MRI experiments using a respiratory probe (SAII Instruments, Stony Brook, NY - USA). After the scout image acquisition, a T_2-weighted (T_{2w}) anatomical image was acquired with a Fast Spin Echo sequence (TR 2500 s; TE 41 ms; number of slices 10; slice thickness 1.5 mm; FOV 40 mm; matrix 128 × 128; four averages; acquisition time 2 m 40 s).

DCE–MRI was performed using an axial 2D T_{1w} spoiled gradient echo sequence (TR 40 ms; TE 1.8 ms; flip angle 60°; number of slices 10; slice thickness 1.5 mm; FOV 40 mm; matrix 128 × 128; one acquisition; temporal resolution 58 s per image) with dynamic series was acquired with three initial pre-contrast T1w images and 47 dynamic post-contrast images for a total examination time of 50 min. The contrast agents were injected intravenously at a dose of 0.05 mmol Gd/kg. Six mice were used in each experimental group for each blood-pool contrast agents.

MR images were evaluated using in-house written software developed in Matlab (MathWorks, Natick, MA) and signal intensities (SI) of tumor, muscle and artery was measured in source images by drawing corresponding ROIs to evaluate SI enhancement following contrast agent injection.

ICP-MS Gd quantification in tumor bearing mice

B16F10 xenografts bearing male C57BL/6 mice (4- to 6-week-old) were used in the study (n = 6 animals per each contrast agent) and injected with a dose of 0.05 mmol Gd/kg intravenously via tail vein. The mice were euthanized at 60 min post-injection. The tumors were collected, wet-weighed and Gadolinium concentrations were measured by ICP-MS. The percentage of injected dose per gram (%ID/g) was determined for each contrast agent inside tumors. The averages and standard deviations were determined.

RESULTS

The chemical structures of the three Gd agents used in this work, namely MS-325 (Vasovist or Ablavar), B22956/1 and Gd-AAZTA-MADEC are reported in **Fig. 1**. In the same figure the structure of B25716/1 is also reported. B25716/1 has been the subject of a recent study and it is strictly analogous to Gd-AAZTA-MADEC [22]. They simply differ for the length of the aliphatic chain (C_4 for B25716/1 and a C_9 for Gd-AAZTA-MADEC, respectively) spacing the targeting deoxycholic acid moiety from the Gd-AAZTA-unit. The elongation of the spacer in Gd-AAZTA-MADEC is expected to remove the steric constrain that causes the decrease of the number of water molecules in B25716/1 (q=1) upon albumin binding [22], in respect to the parent Gd-AAZTA complex (q=2).

Synthesis

The preparation of Gd-AAZTA-MADEC is sketched in **Scheme 1**. The starting materials are the protected bifunctional derivative **1**, recently prepared through a chemo-enzymatic approach [24], and the methyl ester of 3-aminodeoxycholic acid, the latter prepared from deoxycholic acid according to a procedure reported by Anelli et al. [25]. Standard amide coupling between **1** and **2** using DCC/DMAP in dichloromethane yields the protected conjugate **3** in 64% yield. Removal of the methyl ester and *t*-butyl ester groups is best obtained by a sequential combination of a mild alkaline hydrolysis (to avoid the cleavage of the amide bond) followed by the addition of trifluoroacetic acid in dichloromethane, both performed at room temperature. The obtained ligand AAZTA-MADEC is then reacted with freshly prepared $Gd(OH)_3$ to give the desired Gd-AAZTA-MADEC chelate.

Relaxometric characterization

The presence of the long aliphatic chain between the heptadentate ligand AAZTA and the deoxycholic acid moiety further prompts the formation of self assembled adducts, thus affecting the relaxation enhancement properties of the Gd-AAZTA-MADEC. The critical concentration for the formation of self-assembled systems was conveniently determined by measuring the ^{1}H relaxation rate as a function of the Gd complex concentration. In the case of Gd-AAZTA-MADEC a sharp variation of the linear slope is observed when the system passes from monomeric state to self assembled systems. From the data reported in **Fig. S1** (Supplementary materials), the turning point occurs at a concentration of 1 mM of Gd-AAZTA-MADEC. At 20MHz and 298K, the relaxivity of Gd-AAZTA-MADEC below this concentration threshold is 13.9 $mM^{-1}s^{-1}$, whereas the relaxivity of the self assembled structure is 20 $mM^{-1}s^{-1}$. The relaxivity of the non assembled form is similar to the one reported for B25716/1 and it is about 35% higher than that of the corresponding DTPA-deoxycholic acid system (named B22956/1), mainly as a consequence of the increased hydration of the Gd ion coordinated to the heptadentate AAZTA ligand (q=2).

From the analysis of the temperature dependence of the transverse relaxation rate of the metal-bound ^{17}O water resonance (**Fig. S2**, Supplementary materials), a τ_M value of 100 ns was obtained at 298K. This value is similar to that reported for the parent Gd-AAZTA complex (90 ns [27]) and shorter than the ones reported for B22956/1 (122 ns) and for B25716/1 (195 ns). It is in the optimal range for the attainment of high relaxivity in the presence of long molecular reorientational times [28, 29].

Gd-AAZTA-MADEC was stable in human serum at 37°C for 6 hours with no release of Gd^{3+} cation. Less than 1.2% changes in R_1 values were observed up to 6 h of incubation (**Fig. S3**, Supplementary materials).

Binding to albumin

Owing to the possible use as an angiographic MRI system, the interaction with the human serum albumin has been investigated in detail. The water proton relaxation rates of solutions containing the paramagnetic complex were measured in the presence of increasing concentrations of the serum protein (proton relaxation enhancement method) [22]. With the estimation of the binding strength (nK_A, where n is the number of binding sites and K_A the thermodynamic association constant) these

measurements provide a direct assessment of the relaxivity of the macromolecular adduct (r_1^b). Analysis of the relaxometric data obtained at 0.47T from the titration of a 60 μM aqueous solution of the Gd complex with HSA at pH 7.4 and 298 K (**Fig. 2A**) allowed to determine the nK_A and r_1^b values ($8.9x10^5$ ±5.6 M^{-1} and 38.7 ±0.3 $mM^{-1}s^{-1}$, respectively). The observed binding affinity to HSA is significantly higher than the one reported for B22956/1 ($4.5x10^4$ M^{-1}) [26]. Both nK_A and r_1^b of Gd-AAZTA-MADEC are also markedly higher than the values observed for the strictly related B25716/1 complex (nK_A = $2x10^4$ M^{-1} and r_1^b = 29 $mM^{-1}s^{-1}$, respectively). The observed relaxivity of the HSA-bound complex (r_1^b) is close to the value of 40 $mM^{-1}s^{-1}$ obtained at 20MHz and 298K for the clinical-approved MS-325 [26].

To get more insight into the relaxometric parameters of the supramolecular adduct between Gd-AAZTA-MADEC and HSA, NMRD profiles have been recorded in the range of 0.01-70 MHz at 289K in phosphate buffer solution (**Fig. 2B**). The NMRD profile shows high relaxivity values at all fields, with a marked peak centered at 30 MHz, typical of slowly tumbling systems. The observed r_1 values suggest that, upon binding to the protein, Gd-AAZTA-MADEC maintains the two inner sphere water molecules. The data were fitted to the Lipari-Szabo model-free approach that takes into account the presence of motions due to internal rotations, characterized by a correlation time τ_{RL}, superimposed to the global motion described in term of the correlation time τ_{RG}. [30] The results are collected in **Table 1** and the fitting reveals a long global (whole adduct) rotational correlation time (τ_{RG}= 6 ns) and a short local rotational time (τ_{RL}=460 ps). The order factor value S^2 obtained from the fitting was founded to be 0.58, supporting the view that flexible movements of Gd-AAZTA-MADEC occur at the binding site.

To probe the HSA binding interaction more in depth, competition assays with specific molecules, like warfarin (site I) and ibuprofen (site II) were carried out [31]. However, since drug binding site I on HSA is a very large binding domain, with three distinct subdomains, which can accommodate simultaneously small-to-medium size molecules, it has been deemed useful to probe the drug binding site I also with iodipamide in addition to warfarin. In fact, there is the possibility that this binding site can accommodate the Gd-containing molecule without displacing warfarin. Iodipamide is a molecule larger than warfarin, thus yielding additional information on the characteristics of the binding mode of Gd-AAZTA-MADEC. In a typical competition assay, a solution containing a Gd complex and HSA (in a ratio such that the Gd complex/ HSA adduct is > 50%) undergoes T_1 measurements (at 20 MHz and 298K) in the presence of increasing amounts of the competitor molecule. A decrease of the R_{10bs} is an indication of the competition between the Gd-containing probe and the added substrate for the same binding site on the protein. In **Fig. 2C** the results for the

competition-binding assay between Gd-AAZTA-MADEC and classical HSA binding substrates is reported and compared with that of B22956/1 and MS-325. For Gd-AAZTA-MADEC, only with iodipamide we observed a marked decrease in the R_{1Obs}, whereas no effect is obtained in the case of ibuprofen and warfarin. B22956/1 showed a similar displacement from site I when using iodipamide, suggesting a strong interaction with site I, as for Gd-AAZTA-MADEC. On the contrary, a completely different behavior was observed in the case of MS-325 with showed binding interactions at both site I and site II. In fact, for MS-325 both ibuprofen and iodipamide induced a comparable displacement, which was not previously observed, likely due to the use of the smaller warfarin molecule [32].

Comparison of the relaxation enhancements in human and murine serum albumins

Fig. 3 reports the high field relaxivity values obtained for Gd-AAZTA-MADEC, MS-325 and B22956/1 (0.5 mM) at 37°C in the presence of human and murine serum albumin, respectively. As the relaxivity peak at ca. 1 T is determined by the formation of the supramolecular adduct with the paramagnetic complex, it was deemed of interest to compare human vs murine serum albumins in order to extract some insight into the species-related albumin binding of the Gd(III) complexes herein considered. Under the applied experimental conditions, the already high relaxivity shown by Gd-AAZTA-MADEC in human serum albumin (r_{1p}^{bound} = 31 $mM^{-1}s^{-1}$) was even higher when added to the mouse serum albumin (r_{1p}^{bound} = 40 $mM^{-1}s^{-1}$, **Fig. 3A**). Conversely, MS-325 displayed a dramatic decrease of the relaxation enhancement from ca. 39 $mM^{-1}s^{-1}$ with human serum albumin (at 40 MHz and 37°C) to 16 $mM^{-1}s^{-1}$ with mouse serum albumin (**Fig. 3B**). For B22956/1 there is almost perfect overlap between the relaxation enhancements values observed in the two species, with r_{1p}^{bound} of 19 $mM^{-1}s^{-1}$ and 22 $mM^{-1}s^{-1}$ for human and mouse serum albumin, respectively (**Fig. 3C**).

Pharmacokinetic and biodistribution results

Measurements of the average plasma Gd(III) concentrations as percentage of the initial dose (C_0) following a single bolus injection through the tail vein at a dose of 0.05 mmol Gd/kg revealed a prolonged blood residence of Gd-AAZTA-MADEC as compared to B22956/1 and MS-325 (**Fig. 4**). The average plasma Gd concentration of Gd-AAZTA-MADEC at 5 min post-injection was 32.8% of the initial dose, while that of B22956/1 and MS-325 was 14.8% and 18.9%, respectively. The

blood concentration gradually decreased with time for all the Gd-based contrast agents and at 6 h post-injection the plasma Gd concentration was less than 1%. The Gd(III) plasma concentration profiles of the agents were analyzed with a two-compartment pharmacokinetic model. The mean values of half-life associated with the elimination phase, $t_{1/2\beta}$, was longer for Gd-AAZTA-MADEC (32.6 min) than for MS-325 (29.1 min), and for B22956/1 (23.7 min). The elimination half-lives of B22956/1 and MS-325 are comparable to previous values obtained in rats [20, 33].

Biodistributions of Gd-AAZTA-MADEC in normal BALB/c mice at 24 h after intravenous injection of Gd-AAZTA-MADEC at a dose of 0.05 mmol Gd/kg are shown in **Fig. S4** (Supplementary materials). At 1 days post-injection, a reduced amounts of Gd-AAZTA-MADEC remained in the liver and in the spleen (%ID/g = 7.9 ±1.3 and 2.7 ±0.3 for liver and spleen, respectively), likely reflecting a delayed accumulation owing to the hydrophobicity properties. Only a negligible amount of Gd was retained in the other organs analyzed.

Magnetic Resonance Angiography

Fig. 5 shows representative three-dimensional maximum intensity projection (MIP) contrast enhanced images of mice i.v. treated with Gd-AAZTA-MADEC, B22956/1, MS-325 and B25716/1 at 2, 5, 10, 15, 30, 45 and 60 min post-injection of the contrast agents at a dose of 0.05 mmol Gd(III) / kg. The four investigated CAs showed different contrast enhancement properties, either in terms of persistence or of localization of the contrast. Gd-AAZTA-MADEC resulted in stronger and more prolonged contrast enhancement than B22956/1, B25716/1 and MS-325 in the heart and blood vessels. The visualization of vessels resulted well enhanced up to 30 min post-injection, whereas MS-325 resulted in significant blood pool enhancement only at 2 min post-injection, with the signal that gradually faded away due to the clearance of the agent from the blood, resulting in a strong enhancement inside the bladder which gradually increased over time. B22956/1 exhibited longer vascular enhancement duration than MS-325, but lower then Gd-AAZTA-MADEC. B22956/1 also resulted in significant enhancement in the liver and gallbladder. B25716/1 showed comparable enhancement of heart, vessels and liver just upon administration, with a constant accumulation inside the liver, similarly to B22956/1.

Contrast Enhanced-MRI of tumor-bearing mice

Fig. 6A shows the dynamic signal intensity enhancement in C57BL/6 mice transplanted subcutaneously with B16F10 melanoma cells measured in the tumor region. Significant contrast enhancement was observed, already at 10 min post-injection, for Gd-AAZTA-MADEC, B22956/1, MS-325 and B25716/1, of 1.72, 0.91, 1.03 and 1.50, respectively. The contrast enhancement slowly reduced thereafter for B22956/1 and B25716/1, (0.62 and 0.53 for B22956/1 and 1.40 and 1.31 for B25716/1, at 30 and 45 min, respectively), while for Gd-AAZTA-MADEC and MS-325 remained constant along time (1.77 and 1.78 for Gd-AAZTA-MADEC and 1.06 and 1.04 for MS-325, at 30 and 45 min, respectively).

Post-cull analysis of tumor accumulation demonstrated that approximately 2.1 ±0.1% of the injected dose (0.05 mmol Gd / kg) of Gd-AAZTA-MADEC had accumulated per gram of tumor, during the 1 h following intravenous administration (**Fig. 6B**). This proved to be a significant ($p<0.05$) and substantial increase compared to B22956/1 (%ID/g = 0.2 ±0.01%), MS-325 (%ID/g = 0.7 ±0.4%) and B25716/1 (%ID/g = 1.0 ±0.03%). The levels of Gd accumulation within the tumor are in accordance with the changes in T_1 values, at the same time point after injection (60 min), as measured with the MRI-based approach (**Fig. S5**, Supplementary materials). Accumulation of Gd-AAZTA-MADEC within the tumor results in a marked T_1 shortening, in comparison to the lower extent of T_1 reduction for all the other investigated blood-pool agents.

All the investigated CAs induced a good contrast enhancement in the tumor region, thanks to the enhanced permeability retention effect, at the investigated doses of 0.05 mmol Gd/kg (**Fig. 7A-D**). However, Gd-AAZTA-MADEC showed the highest contrast enhancement inside tumor region, providing a better visualization of intratumoral heterogeneity.

DISCUSSION

In the last two decades it has been widely recognized the potential of blood pool Gd-based contrast agent in MRI investigations. In particular, at pre-clinical level, several reversible human serum albumin binding complexes have shown to be particularly useful to assess therapeutic response to different therapeutic protocols using the DCE-MRI based procedures [34]. Several issues, including contrast efficiency, overall stability, vascular retention time, extravasation properties, as well as magnetic field and species dependence have to be considered for an optimal design of an albumin-binding agent for vasculature and tumor imaging. In this study, we addressed all these properties to evaluate a new blood pool contrast agent, Gd-AAZTA-MADEC, by comparing its contrast

enhancement capabilities against two well established Gd-based vascular agents, namely B22956/1 and MS-325, and with B25716/1.

One of the key factor for obtaining an optimal blood pool agent is the binding affinity toward HSA, that occurs through the best matching with the two main binding cavities of the protein (Sudlow's drug site I and site II [35, 36]). An increase of the binding affinity can be pursued by a correct balance between the targeting moiety that penetrates inside the hydrophobic pocket and the length of the spacer that has to be long enough to allow the Gd coordinating cage to protrude outside the protein surface. However, at the same time, the spacer should be short enough to prevent local internal rotational motions that could be detrimental to the attainable relaxation enhancement [37-39]. On this basis, Gd-AAZTA-MADEC was designed by elongating the spacer by a C5 unit in respect to B25716/1. This minor modification has brought to increase the binding affinity by more than one order of magnitude (nK_A = 8.9x10^5 and nK_A = 2.0x10^4 for Gd-AAZTA-MADEC and B25716/1, respectively). Gd-AAZTA-MADEC, B22956/1 and B25716/1 share the same targeting moiety, represented by a 3-aminodeoxycholic residue, but linked to the coordination cage through a flexible spacer made of an aliphatic chain of different length. On the basis of the observed *nKA* values towards human serum albumin, one can conclude that the longer the spacer, the higher the binding affinity (**Table 1**).

The prolonged blood circulation time can be similarly explained by the higher binding affinity constant to HSA that in turn results in a reduced amount of the free form that is excreted by kidneys. In addition, Gd-AAZTA-MADEC showed a reduced liver uptake, at early time points post-injection, in comparison to B22956/1 and to B25716/1, and a reduced renal filtration, as compared to MS-325, therefore resulting in higher blood concentration values at all the investigated time points. To confirm the different pharmacokinetic fate of the investigated blood pool agents, signal intensities (normalized to the signal intensity of the muscle region) were measured in several organs, such as heart, liver, urinary bladder and gall bladder, following i.v. injection (**Fig. S6**, Supplementary materials**)**. The heart contrast enhancement was significant higher for Gd-AAZTA-MADEC up to 30 min, showing a prolonged and higher contrast enhancement in the blood pool as compared to B25716/1, MS-325 and B22956/1, respectively. For the same reason Gd-AAZTA-MADEC resulted in a clearer visualization of small blood vessels, providing effective contrast enhancement for more than 15 min (**Fig. 5**). The contrast enhancement pattern of the agents in the blood pool was consistent to the plasma pharmacokinetic results. In addition, the effective enhancement window of Gd-AAZTA-MADEC in humans may be longer than in mice because the blood circulation in humans is much slower than in mice. Consequently, considering that

elimination half-lives in humans are six to nine fold longer than in mice, these results indicated that the one order of magnitude higher affinity constant to HSA for Gd-AAZTA-MADEC may allow robust steady-state MRI angiographic acquisitions [40]. Beyond angiography, recent studies have utilized MS-325 as a biomarker for tissue albumin concentration for assessing reperfused myocardial infarction [41]. The design of a new blood pool agent with optimized albumin binding properties may allow further improvements of this new clinical indication.

Besides the binding affinity, the number of the coordinated water molecules to the Gd ion plays a key role because the relaxivity increases with the hydration state. Obviously, the enhanced hydration cannot occur at the expenses of the overall thermodynamic stability, which is one of the main prerequisites for a metal complex to be considered for *in vivo* application [42, 43]. MS-325, the only FDA-approved blood-pool CA, has a coordination cage with q =1, similar to B22956/1, while the proposed Gd-AAZTA-MADEC owns two coordinated water molecules, therefore enabling higher relaxivity. At the same time, the stability of the AAZTA cage has been shown to be sufficiently high to prevent the release of the Gd(III) ion [44]. The hydration number of two was maintained upon binding to HSA, despite the relaxivity of the bound complex not being as high as one may expect on the basis of the established theory. One reason to account for the observed behaviour could be correlated to the high flexibility of the spacer that yields a certain degree of local motion at the binding site, in turn resulting in a shortening of the effective τ_{RL}. In the comparison with B25716/1 [22], for which the interaction to HSA causes the reduction of the inner-sphere water molecules, Gd-AAZTA-MADEC, thanks to the longer spacer, succeeds to maintain the pristine number of coordinating water molecules and the relaxivity of the HSA-adduct sensibly higher than those obtained for the analogous bile acid derivatives.

It is well established that the Gd-based CAs present a field-dependent relaxivity which may show a remarkable enhancement at fields of 0.5-1.5T in the presence of slow molecular reorientation [28, 45-48]. Recently, the development of preclinical MRI scanners at 1T attracted new attention towards the advantages associated to the combination of macromolecular Gd-based systems and low field scanners [8]. Vascular imaging requires both sufficient high relaxivity and long blood half life to obtain high contrast enhanced steady state images. Previous studies from Caravan et al. already showed species dependence on albumin binding [49, 50]. As a consequence, a blood pool CA that has been optimized for humans, may show sub-optimal relaxivity properties when used in other species. This marked species dependence of the investigated blood pool CAs explains the differences in contrast enhancement capabilities in the corresponding angiographic images. The combination of the longest elimination half life, with the highest relaxivity showed by Gd-AAZTA-

MADEC in mouse serum, resulted in the brightest and prolonged MIP angiographic images. MS-325, despite a slightly shorter elimination half-life in comparison to Gd-AAZTA-MADEC, it is not so suited for mice angiographic images due to its low relaxation efficiency in mice serum. Similarly, B22956/1 and B25716/1 despite having high relaxivity in mouse serum, resulted in angiographic images that are not optimal, due to the relative fast elimination kinetic and to the high liver uptake. DCE-MRI is a promising method for characterizing tumor angiogenesis and tumor response to antiangiogenic treatment [51, 52]. The accuracy of tumor microvasculature measurements relies, besides other factors, on the pharmacokinetic properties of the injected tracer and on its capabilities to induce marked signal intensities changes on sequential magnetic resonance images. Several macromolecular blood pool agents have been investigated, from small Gd-based complexes with reversible binding to serum albumin, to Gd-complexes covalently conjugated to albumin, to dendrimers or to biocompatible hyaluronan scaffold [53-56]. A relevant issue is often related to their biological fate, where significant liver and spleen accumulation is usually observed as consequence of their higher molecular weight and/or lipophilicity, resulting in a reduced accumulation inside tumor region. Our findings showed that the tumor contrast enhancement of Gd-AAZTA-MADEC was the highest among B22956/1, B25716/1 and MS-325 during all the dynamic acquisition series. The slow decrease of contrast enhancement shown by B22956/1 may be likely due to the accumulation of the contrast agent in the liver and gall-bladder, thus resulting in a lower blood pool concentration along time, as accounted by the pharmacokinetic profile, which turns in a reduced extravasation to the tumor region. On the contrary, the prolonged vascular retention for Gd-AAZTA-MADEC, allows a continued accumulation of the macromolecular adduct inside the tumor, thus resulting in a sustained and elevated contrast enhancement and Gd accumulation. Hence, the Gd-AAZTA-MADEC capability to accumulate inside tumor regions may be exploited for an accurate characterization of tumor microvasculature [57].

CONCLUSION

In summary, our study demonstrates that Gd-AAZTA-MADEC is a novel blood-pool contrast MR agent with optimal pharmacokinetic and relaxometric properties, showing significant enhancement of the vasculature and extravasation inside tumor for a prolonged period of time. Based on these results, preclinical benchtop MRI scanners working at 0.5-1.5 T may benefit from the use of this improved blood pool contrast agent to establish DCE-MRI as a tool for routine preclinical imaging as well as in combination with multimodality imaging scanners [58].

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References

1. Gillies RJ, Raghunand N, Karczmar GS, Bhujwalla ZM (2002) MRI of the tumor microenvironment. J Magn Reson Imaging 16:430-450.
2. Kiessling F, Jugold M, Woenne EC, Brix G (2007) Non-invasive assessment of vessel morphology and function in tumors by magnetic resonance imaging. Eur Radiol 17:2136-2148.
3. Aime S, Barge A, Botta M, Terreno E (2003) Interactions of lanthanides and their complexes with proteins. Conclusions regarding magnetic resonance imaging. Met Ions Biol Syst 40:643-682.
4. Jung KH, Kim HK, Lee GH, et al. (2011) Gd complexes of macrocyclic diethylenetriaminepentaacetic acid (DTPA) biphenyl-2,2'-bisamides as strong blood-pool magnetic resonance imaging contrast agents. J Med Chem 54:5385-5394.
5. Boros E, Karimi S, Kenton N, Helm L, Caravan P (2014) Gd(DOTAlaP): exploring the boundaries of fast water exchange in gadolinium-based magnetic resonance imaging contrast agents. Inorg Chem 53:6985-6994.
6. Yang CT, Chandrasekharan P, He T, et al. (2014) An intravascular MRI contrast agent based on Gd(DO3A-Lys) for tumor angiography. Biomaterials 35:327-336.
7. Aime S, Gianolio E, Longo D, Pagliarin R, Lovazzano C, Sisti M (2005) New insights for pursuing high relaxivity MRI agents from modelling the binding interaction of Gd(III) chelates to HSA. Chembiochem 6:818-820.
8. Geninatti-Crich S, Szabo I, Alberti D, Longo D, Aime S (2011) MRI of cells and mice at 1 and 7 Tesla with Gd-targeting agents: when the low field is better! Contrast Media Mol Imaging 6:421-425.
9. Gossmann A, Okuhata Y, Shames DM, et al. (1999) Prostate cancer tumor grade differentiation with dynamic contrast-enhanced MR imaging in the rat: comparison of macromolecular and small-molecular contrast media--preliminary experience. Radiology 213:265-272.
10. Marzola P, Degrassi A, Calderan L, et al. (2005) Early antiangiogenic activity of SU11248 evaluated in vivo by dynamic contrast-enhanced magnetic resonance imaging in an experimental model of colon carcinoma. Clin Cancer Res 11:5827-5832.
11. O'Connor JP, Jackson A, Parker GJ, Jayson GC (2007) DCE-MRI biomarkers in the clinical evaluation of antiangiogenic and vascular disrupting agents. Br J Cancer 96:189-195.
12. Roberts TP, Turetschek K, Preda A, et al. (2002) Tumor microvascular changes to anti-angiogenic treatment assessed by MR contrast media of different molecular weights. Acad Radiol 9 Suppl 2:S511-513.
13. Turetschek K, Preda A, Novikov V, et al. (2004) Tumor microvascular changes in antiangiogenic treatment: assessment by magnetic resonance contrast media of different molecular weights. J Magn Reson Imaging 20:138-144.
14. Caravan P, Ellison JJ, McMurry TJ, Lauffer RB (1999) Gadolinium(III) Chelates as MRI Contrast Agents: Structure, Dynamics, and Applications. Chem Rev 99:2293-2352.
15. Botta M, Avedano S, Giovenzana GB, et al. (2011) Relaxometric Study of a Series of Monoaqua Gd-III Complexes of Rigidified EGTA-Like Chelators and Their Noncovalent Interaction with Human Serum Albumin. European Journal of Inorganic Chemistry:802-810.
16. Avedano S, Tei L, Lombardi A, et al. (2007) Maximizing the relaxivity of HSA-bound gadolinium complexes by simultaneous optimization of rotation and water exchange. Chem Commun (Camb):4726-4728.
17. Ferreira MF, Martins AF, Martins JA, Ferreira PM, Toth E, Geraldes CF (2009) Gd(DO3A-N-alpha-aminopropionate): a versatile and easily available synthon with optimized water exchange for the synthesis of high relaxivity, targeted MRI contrast agents. Chem Commun (Camb):6475-6477.

18. Elemento EM, Parker D, Aime S, Gianolio E, Lattuada L (2009) Variation of water exchange dynamics with ligand structure and stereochemistry in lanthanide complexes based on 1,4-diazepine derivatives. Org Biomol Chem 7:1120-1131.
19. Strauch RC, Mastarone DJ, Sukerkar PA, Song Y, Ipsaro JJ, Meade TJ (2011) Reporter protein-targeted probes for magnetic resonance imaging. J Am Chem Soc 133:16346-16349.
20. Parmelee DJ, Walovitch RC, Ouellet HS, Lauffer RB (1997) Preclinical evaluation of the pharmacokinetics, biodistribution, and elimination of MS-325, a blood pool agent for magnetic resonance imaging. Invest Radiol 32:741-747.
21. Cavagna FM, Lorusso V, Anelli PL, Maggioni F, de Haen C (2002) Preclinical profile and clinical potential of gadocoletic acid trisodium salt (B22956/1), a new intravascular contrast medium for MRI. Acad Radiol 9 Suppl 2:S491-494.
22. Gianolio E, Cabella C, Colombo Serra S, et al. (2014) B25716/1: a novel albumin-binding Gd-AAZTA MRI contrast agent with improved properties in tumor imaging. J Biol Inorg Chem 19:715-726.
23. Anelli PL, Brocchetta M, Lattuada L, et al. (2009) Scale-Up of Trisodium [(3 beta,5 beta,12(alpha)-3-[[4(S)-4-[Bis[2-[bis[(carboxy-kO)methyl]amino-kN]ethyl]amino-kN]-4-(carboxy-kO)-1-oxabutyl]amino]-12-hydroxycholan-24-oato(6-)]gadolinate(3-)], a Gd(III) Complex under Development As a Contrast Agent for MRI Coronary Angiography. Organic Process Research & Development 13:739-746.
24. Minazzi P, Lattuada L, Menegotto IG, Giovenzana GB (2014) An enzymatic approach to bifunctional chelating agents. Org Biomol Chem 12:6915-6921.
25. Anelli PL, Lattuada L, Uggeri F (1998) One-pot Mitsunobu-Staudinger preparation of 3-aminocholan-24-oic acid esters from 3-hydroxycholan-24-oic acid esters. Synthetic Communications 28:109-117.
26. Aime S, Chiaussa M, Digilio G, Gianolio E, Terreno E (1999) Contrast agents for magnetic resonance angiographic applications: 1H and 17O NMR relaxometric investigations on two gadolinium(III) DTPA-like chelates endowed with high binding affinity to human serum albumin. J Biol Inorg Chem 4:766-774.
27. Aime S, Calabi L, Cavallotti C, et al. (2004) [Gd-AAZTA](-): A new structural entry for an improved generation of MRI contrast agents. Inorganic Chemistry 43:7588-7590.
28. Sherry AD, Caravan P, Lenkinski RE (2009) Primer on gadolinium chemistry. J Magn Reson Imaging 30:1240-1248.
29. Werner EJ, Datta A, Jocher CJ, Raymond KN (2008) High-relaxivity MRI contrast agents: where coordination chemistry meets medical imaging. Angew Chem Int Ed Engl 47:8568-8580.
30. Andrec M, Montelione GT, Levy RM (1999) Estimation of dynamic parameters from NMR relaxation data using the Lipari-Szabo model-free approach and Bayesian statistical methods. J Magn Reson 139:408-421.
31. Curry S (2009) Lessons from the crystallographic analysis of small molecule binding to human serum albumin. Drug Metab Pharmacokinet 24:342-357.
32. Caravan P, Parigi G, Chasse JM, et al. (2007) Albumin binding, relaxivity, and water exchange kinetics of the diastereoisomers of MS-325, a gadolinium(III)-based magnetic resonance angiography contrast agent. Inorg Chem 46:6632-6639.
33. de Haen C, Anelli PL, Lorusso V, et al. (2006) Gadocoletic acid trisodium salt (b22956/1): a new blood pool magnetic resonance contrast agent with application in coronary angiography. Invest Radiol 41:279-291.
34. Dirksen MS, Lamb HJ, Kunz P, Robert P, Corot C, de Roos A (2003) Improved MR coronary angiography with use of a new rapid clearance blood pool contrast agent in pigs. Radiology 227:802-808.
35. Fasano M, Curry S, Terreno E, et al. (2005) The extraordinary ligand binding properties of human serum albumin. IUBMB Life 57:787-796.
36. Aime S, Botta M, Fasano M, Crich SG, Terreno E (1996) Gd(III) complexes as contrast agents for magnetic resonance imaging: A proton relaxation enhancement study of the interaction with human serum albumin. Journal of Biological Inorganic Chemistry 1:312-319.
37. Gianolio E, Giovenzana GB, Longo D, Longo I, Menegotto I, Aime S (2007) Relaxometric and modelling studies of the binding of a lipophilic Gd-AAZTA complex to fatted and defatted human serum albumin. Chemistry 13:5785-5797.
38. Avedano S, Botta M, Haigh JS, Longo DL, Woods M (2013) Coupling fast water exchange to slow molecular tumbling in Gd3+ chelates: why faster is not always better. Inorg Chem 52:8436-8450.

39. Thompson MK, Misselwitz B, Tso LS, Doble DM, Schmitt-Willich H, Raymond KN (2005) In vivo evaluation of gadolinium hydroxypyridonate chelates: initial experience as contrast media in magnetic resonance imaging. J Med Chem 48:3874-3877.
40. Aime S, Caravan P (2009) Biodistribution of gadolinium-based contrast agents, including gadolinium deposition. J Magn Reson Imaging 30:1259-1267.
41. Richardson OC, Bane O, Scott ML, et al. (2014) Gadofosveset-based biomarker of tissue albumin concentration: Technical validation in vitro and feasibility in vivo. Magn Reson Med.
42. Laurent S, Elst LV, Muller RN (2006) Comparative study of the physicochemical properties of six clinical low molecular weight gadolinium contrast agents. Contrast Media Mol Imaging 1:128-137.
43. Baranyai Z, Palinkas Z, Uggeri F, Maiocchi A, Aime S, Brucher E (2012) Dissociation Kinetics of Open-Chain and Macrocyclic Gadolinium(III)-Aminopolycarboxylate Complexes Related to Magnetic Resonance Imaging: Catalytic Effect of Endogenous Ligands. Chemistry-a European Journal 18:16426-16435.
44. Baranyai Z, Uggeri F, Giovenzana GB, Benyei A, Brucher E, Aime S (2009) Equilibrium and Kinetic Properties of the Lanthanoids(III) and Various Divalent Metal Complexes of the Heptadentate Ligand AAZTA. Chemistry-a European Journal 15:1696-1705.
45. Zhang Z, Greenfield MT, Spiller M, McMurry TJ, Lauffer RB, Caravan P (2005) Multilocus binding increases the relaxivity of protein-bound MRI contrast agents. Angew Chem Int Ed Engl 44:6766-6769.
46. Manus LM, Mastarone DJ, Waters EA, et al. (2010) Gd(III)-nanodiamond conjugates for MRI contrast enhancement. Nano Lett 10:484-489.
47. Song Y, Kohlmeir EK, Meade TJ (2008) Synthesis of multimeric MR contrast agents for cellular imaging. J Am Chem Soc 130:6662-6663.
48. Datta A, Hooker JM, Botta M, Francis MB, Aime S, Raymond KN (2008) High relaxivity gadolinium hydroxypyridonate-viral capsid conjugates: nanosized MRI contrast agents. J Am Chem Soc 130:2546-2552.
49. Zech SG, Eldredge HB, Lowe MP, Caravan P (2007) Protein binding to lanthanide(III) complexes can reduce the water exchange rate at the lanthanide. Inorg Chem 46:3576-3584.
50. Eldredge HB, Spiller M, Chasse JM, Greenwood MT, Caravan P (2006) Species dependence on plasma protein binding and relaxivity of the gadolinium-based MRI contrast agent MS-325. Invest Radiol 41:229-243.
51. Jackson A, O'Connor JP, Parker GJ, Jayson GC (2007) Imaging tumor vascular heterogeneity and angiogenesis using dynamic contrast-enhanced magnetic resonance imaging. Clin Cancer Res 13:3449-3459.
52. Longo DL, Dastru W, Consolino L, et al. (2015) Cluster analysis of quantitative parametric maps from DCE-MRI: application in evaluating heterogeneity of tumor response to antiangiogenic treatment. Magn Reson Imaging 33:725-736.
53. Gugliotta G, Botta M, Tei L (2010) AAZTA-based bifunctional chelating agents for the synthesis of multimeric/dendrimeric MRI contrast agents. Org Biomol Chem 8:4569-4574.
54. Lim J, Turkbey B, Bernardo M, et al. (2012) Gadolinium MRI contrast agents based on triazine dendrimers: relaxivity and in vivo pharmacokinetics. Bioconjug Chem 23:2291-2299.
55. Zhu W, Artemov D (2011) Biocompatible blood pool MRI contrast agents based on hyaluronan. Contrast Media Mol Imaging 6:61-68.
56. Boschi F, Marzola P, Sandri M, et al. (2008) Tumor microvasculature observed using different contrast agents: a comparison between Gd-DTPA-Albumin and B-22956/1 in an experimental model of mammary carcinoma. MAGMA 21:169-176.
57. Bandini S, Curcio C, Macagno M, et al. (2013) Early onset and enhanced growth of autochthonous mammary carcinomas in C3-deficient Her2/neu transgenic mice. Oncoimmunology 2:e26137.
58. Schmid A, Schmitz J, Mannheim JG, et al. (2013) Feasibility of sequential PET/MRI using a state-of-the-art small animal PET and a 1 T benchtop MRI. Mol Imaging Biol 15:155-165.

Figure Legends

Fig. 1. Chemical structures of the investigated blood pool molecules: **(A)** MS-325, **(B)** B22956/1, **(C)** Gd-AAZTA-MADEC, **(D)** B25716/1.

Scheme 1. Synthetic procedure for the preparation of Gd-AAZTA-MADEC.

Fig. 2. (A) Plot of variation of observed longitudinal water proton relaxation rate of a 60 μM solution of Gd-AAZTA-MADEC measured at 0.47T and 25°C. **(B)** $1/T_1$ NMRD profile of 1 mM solution of Gd-AAZTA-MADEC in phosphate buffer solution (□) and bound to HSA (■), measured at 25°C. **(C)** Variation of proton relaxation rates of aqueous solutions of 0.5 mM HSA and 0.05 mM of Gd-AAZTA-MADEC, B22956/1 and MS-325 as a consequence of the competition for the binding to the protein upon addition of the substrates iodipamide and warfarin for site I and ibuprofen for site II ([Competitors]= 5 mM).

Fig. 3. NMRD profiles in presence of mouse serum albumin (open square) and human serum albumin (closed square) at 37°C for **(A)** Gd-AAZTA-MADEC, **(B)** MS-325 and **(C)** B22956/1.

Fig. 4. Plasma level decay as % of the initial dose (C_0) after single intravenous injection of Gd-AAZTA-MADEC (circles), MS-325 (triangles) and B22956/1 (squares) at a dose of 0.05 mmol Gd/kg.

Fig. 5. Whole body coronal maximum intensity projection (MIP) of the 3D FLASH images obtained from the mice after intravenous injection of Gd-AAZTA-MADEC, MS-325, B22956/1and B25716/1(from top to bottom) at a dose of 0.05 mmol Gd/kg. Post-contrast images at 2, 5, 10, 15, 30, 45 and 60 min are shown here after the subtraction of the corresponding pre-contrast images.

Fig. 6. (A) Time course of MRI signal intensity enhancement in tumor of mice with intravenous injection of Gd-AAZTA-MADEC (circles), MS-325 (triangles), B22956/1 (squares) and B25716/1 (diamond) at a dose of 0.05 mmol Gd/kg. Values are shown as mean ±SD (n=6) for each contrast agent. **(B)** Gd accumulation within tumor tissue at 60 min after injection of Gd-based agents at a dose of 0.05 mmol Gd/kg. The data are expressed as the percentage injected dose per gram tissue (%ID/g) and represented as the mean values of six mice.

Fig. 7. Representative tumor SI enhancement maps overimposed onto anatomical T_{2w} images upon injection of Gd-AAZTA-MADEC **(A)**, B25716/1 **(B)**, MS-325 **(C)** and B22956/1 **(D)** at a dose of 0.05 mmol Gd/kg.

Tables

Table 1. Main relaxometric parameters derived from fitting of ^{1}H-NMRD and $^{17}OR_{2p}$ versus T analysis of Gd-AAZTA-MADEC, and Gd-AAZTA-MADEC bound to HSA.

	$r_{1p\ (20MHz)}$ [mM^{-1}s^{-1}]	Δ^{2}[a] [s^{-2}]	τ_V[b] [ps]	τ_R[c] [ps]		τ_M [f] [ns]	q[g]
Gd-AAZTA-MADEC	13.9	3×10^{19}	27	200		100	2
Gd-AAZTA-MADEC +HSA	38.7	1.55×10^{19}	15.4	τ_{RL}[d]/[ps] 224	τ_{RG}[e]/[ps] 6200	440	2

[a] Squared mean transient zero-field splitting (ZFS) energy. [b] Correlation time for the collision-related modulation of the ZFS Hamiltonian. [c] Re-orientational correlation time.[d] Correlation time for local motion. [e] Correlation time for global motion. [f] Exchange life-time of the coordinated water molecule. [g] Number of inner sphere water molecules.