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Characterization of Estrogen-Receptor-Targeted Contrast-Agents in Solution, Breast Cancer Cells and Tumors in vivo

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Abstract

The estrogen receptor (ER) is a major prognostic biomarker of breast cancer, currently determined in surgical specimens by immunohistochemistry. Two new ER targeted probes, pyridine-tetra-acetate-Gd chelate (PTA-Gd) conjugated either to 17 β -estradiol (EPTA-Gd) or to tamoxifen (TPTA-Gd), were explored as contrast agents for molecular imaging of ER. In solution both probes exhibited a micromolar ER binding-affinity, fast water exchange-rate ($\sim 10^7 \text{s}^{-1}$) and water proton-relaxivity of 4.7 to 6.8 $\text{mM}^{-1}\text{s}^{-1}$. In human breast cancer cells, both probes acted as estrogen agonists and enhanced the water protons T1 relaxation-rate and relaxivity in ER-positive as compared to ER-negative cells, with EPTA-Gd showing a higher ER-specific relaxivity than TPTA-Gd. In studies of breast cancer tumors in vivo EPTA-Gd induced the highest enhancement in ER-positive tumors as compared to ER-negative tumors and muscle tissue, enabling in vivo detection of ER. TPTA-Gd demonstrated the highest enhancement in muscle tissue indicating non specific interaction of this agent with muscle components. The extracellular contrast agents, PTA-Gd and GdDTPA, showed no difference in the perfusion capacity of ER-positive and negative tumors confirming the specific interaction of EPTA-Gd with ER. These findings lay a basis for the molecular imaging of the estrogen receptor using EPTA-Gd as a template for further developments.

Keywords

Molecular MRI; Contrast Agents; Estrogen Receptor; Breast Cancer

Introduction

Breast cancer is the most common malignancy in women, entailing 28% of all female cancers. Despite the advances in early detection and treatment, it is the second leading cause of cancer death among women (1). Imaging technologies that contribute to the earlier detection of breast cancer include mammography, ultrasound and recently, magnetic resonance imaging, which has been proposed for screening high-risk patients (2). The diagnosed lesions are validated and characterized by immunohistochemistry and molecular analysis for profiling breast cancer biomarkers. Both the immunohistochemistry (3–5) and

the genes microarray profiling (6–8) are commonly based on expression of estrogen receptor α (ER) and progesterone receptor (PR) (9–12), Her2 receptor (13–15) and various cytokeratins (16,17).

The fraction of breast cancers with over-expressed ER in women older than 45 years of age is high (~70%) as compared to the other biomarkers (9,18). This makes the ER a most clinically important prognostic marker and predictive marker for hormonal therapy with drugs that inhibit estrogen binding to ER or reduce serum estrogen levels (19, 20). ER level is determined in biopsy or surgical specimens of breast cancer by enzyme-binding immunoassay or immunohistochemistry (3–5,24). These methods sample a small fraction of the tumor, are semi-quantitative, and lack standardization across laboratories (25). In the last 20 years methods in nuclear medicine such as PET (positron emission tomography) and SPECT (single photon emission tomography) have been applied to detect and assess the functional ER status in breast cancer patients by developing new radiolabeled ER- targeted ligands ((26,27) and references cited therein). ^{18}F -fluoroestradiol has been the leading PET agent for imaging ER expression providing quantitative assessment and good correlation with immunohistochemistry results (28).

Here we describe the solution, cellular and in vivo characteristics of new ER targeted MRI contrast agents. Most conventional MRI contrast agents used in clinical practice are Gd-based, low molecular weight, extracellular paramagnetic complexes (e.g. GdDTPA, GdDOTA) that diffuse between the intravascular and interstitial space and are rapidly excreted by the kidneys. These agents measure the transcapillary transfer rate constants and assess the perfusion capacity of the microvasculature network (29). MRI contrast agents targeted to blood components or to extracellular and outer membranal components, as well as internalized inside cells were synthesized and primarily tested in preclinical studies (30, 31 and references cited therein). These agents contained Gd-based chelates attached to low or high molecular weight molecules or were based on iron-oxide nano-particles conjugated to target recognition moieties (32,33 and references cited therein). A different approach to contrast enhancement using paramagnetic shift reagents and chemical exchange saturation transfer (PARACEST) has the potential to report multiple biological interactions in one experiment (34 and references cited therein). Recently new Gd^{3+} chelates designed to detect nuclear receptors such as ER (35,36) or PR (37,38) were synthesized and evaluated as targeted contrast agents. This paper concentrates on describing the nuclear magnetic resonance characteristics of the two ER targeted contrast agents in solution, breast cancer cells and animal model of breast cancer, in vivo.

Methods

Gadolinium chelates targeted to ER

Gd^{3+} -based contrast agents targeted to the estrogen receptor were synthesized and chemically analyzed. These agents are composed of pyridine-tetra-acetate-Gd chelate (PTA-Gd) (39) conjugated by a triple bond either to 17β -estradiol (EPTA-Gd) (35) or to tamoxifen (TPTA-Gd) (36) as shown in Figure 1A.

Cells

Wild type (WT) MDA-MB-231 human breast cancer cells which are ER-negative were obtained from the American Type Culture Collection (Manassas, VA). These cells were genetically engineered to ER-positive cells by transfecting the WT MDA-MB-231 cells with a plasmid encoding tetracycline repressor protein (TR) pcDNA6/TR (T-REx™ system, Invitrogen, Carlsbad, CA) and with a plasmid encoding ER α pcDNA4/ER as previously described (36). The cells were routinely cultured in RPMI medium 1640 supplemented with 10% FCS (Biological Industries, Israel), L-glutamine (2mM), pyruvate (1mM), and 0.1 % combined antibiotics (Bio-Lab, Israel). The expression of ER was induced by adding 1 μ g/ml doxycycline (44577 Doxycycline hyclate, Sigma-Aldrich, St. Louis, MO) to the culture medium.

The effects of EPTA-Gd and TPTA-Gd as compared to those of 17 β - estradiol on the proliferation, c-Myc expression and ER expression were investigated in MCF7 and T47D human breast cancer cells as previously described (40).

Breast cancer xenografts in mice

All experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the Weizmann Institute of Science. Female CB-17 SCID mice (Harlan Biotech Israel Ltd., Israel), 6–7 weeks old, were ovariectomized. After 7 days, the WT (ER-negative) and ER transfected (ER-positive) cells were inoculated (2.5×10^6 cells) into the left and right mammary fat pad, respectively. One week later, ER expression was induced by supplementing the drinking water with 0.2 mg/ml doxycycline (44577 Doxycycline hyclate, Sigma-Aldrich, St. Louis, MO) in 3% sucrose. MRI experiments were conducted 2–4 weeks after cell implantation. During the MRI scanning, mice were anesthetized with Isoflurane (Medeva Pharmaceuticals PA, Inc., Bethlehem, PA) (3% for induction, 1–2% for maintenance) mixed with compressed air (1 liter/min) and delivered through a nasal mask.

MRS/ MRI investigations

Relaxivity in solution—T1 relaxation rate of water protons were measured in phosphate-buffered saline (PBS) solutions containing 0.05 mM to 0.5 mM of either EPTA-Gd or TPTA-Gd at 9.4 T and 15°C (400-DMX spectrometer, Bruker, Karlsruhe, Germany), using an inversion-recovery pulse sequence with 16 time intervals (0 s to 20 s) and relaxation delay of 20 s.

T2 relaxation rate studies in H₂¹⁷O solutions—Variable-temperature T2 relaxation rates of ¹⁷O and ¹H were measured in 5% ¹⁷O-enriched water (Rotem industries, Beer Sheva, Israel) in PBS solutions (pH $\approx$ 6.7) with and without EPTA-Gd and TPTA-Gd. The measurements were conducted at 9.4 T (54.24 MHz for ¹⁷O and 400.13 MHz for ¹H; Bruker 400-DMX spectrometer Karlsruhe, Germany). ¹⁷O and ¹H T2 relaxation rates were measured using a Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence. For ¹⁷O the interval between echoes was 0.2 to 1 ms and for ¹H 5 ms, depending on the T2 relaxation rate.

Studies of cells—MDA-MB-231 cells (ER-positive and ER-negative) were cultured on Biosilon polystyrene beads (160–300 μm , Nunc A/S Roskilde, Denmark) with the standard growth medium for 4 days, and then with phenol-red free medium supplemented with 10% dextran-coated charcoal stripped fetal bovine serum (Biological industries, Beit-Haemek, Israel) for additional 3 days. On day 7 the medium was replaced by serum free medium containing either EPTA-Gd or TPTA-Gd, for 60 min incubation and then the cells were washed with serum free /contrast agent free medium and placed in wells (0.5ml/well) (Microtest 96-well plate, BD Falcon, NJ). Control samples were incubated at all stages and placed in wells with contrast agent free medium. Proton T1 relaxation rates were measured at 23°C with a 4.7 T Bruker Biospec spectrometer (Bruker, Karlsruhe, Germany) equipped with a 4 cm ^1H volume coil, by applying a 2D spin-echo pulse sequence with a field of view (FOV) $8 \times 8 \text{ cm}^2$, matrix of 256×192 , and slice thickness of 3 mm, echo time (TE)=16 ms, and six different repetition times (TRs). T1 relaxation times per pixel was determined by non-linear least-square fitting using simplex algorithm (Matlab R2009b, Mathworks, Natick, MA) of the MRI signal intensity, SI, to the equation $\text{SI} = \text{So}(1 - \exp(-\text{TR}/\text{T1}))$ with two free parameters So and T1.

T1 relaxation rate measurements were also conducted in ER-positive and ER-negative cells cultured on beads as described above and then perfused during the experiments. The cells on beads (2 ml) were transferred to a 10 mm NMR tube that was placed in a 9.4 T NMR spectrometer (DMX-400, Bruker, Karlsruhe, Germany) with a broad-band multi nuclear probe. The cells in the spectrometer were perfused under sterile conditions with oxygenated, phenol- red free and serum-free, medium at $36 \pm 1^\circ\text{C}$ as previously described (41). EPTA-Gd and TPTA-Gd were added at various concentrations to the perfusion medium reservoir. Cell viability during the experiments was monitored by ^{31}P NMR. ^{31}P spectra were recorded at 161.973 MHz by acquiring 900 transients with 45° pulses, 1 s repetition time, spectral width of 34 ppm, with a continuous composite pulse proton decoupling. The chemical shift of ^{31}P signals was referenced to $\alpha\text{-NTP}$ at -10.03 ppm . Spectra were processed with a line broadening of 20 Hz. Proton T1 relaxation rates were measured using an inversion recovery pulse sequence with 16 inversion times (0.05 s to 15 s) and 15 s relaxation delay. T1 relaxation rates, RI , were calculated according to the equation above using non linear least-square Levenberg–Marquardt algorithm (Origin, version 6.1). RI was defined as the difference between RI of cells perfused with medium containing the contrast agent (the contrast agent is present in the intra and extracellular compartments) and RI of these cells perfused with contrast free medium (both intra and extracellular compartments do not contain the contrast agent). T1 relaxivity, rI in $\text{mM}^{-1}\text{s}^{-1}$ was calculated from the slope of a linear fit of RI as a function of the contrast agent concentration.

In vivo contrast enhanced MRI—In vivo MR images were acquired on a 9.4T Biospec AVANCE II spectrometer (Bruker, Karlsruhe, Germany) equipped with a ^1H radiofrequency quadrature volume resonator. Anatomical images were recorded using multi-slice fast T2-weighted sequence with TE/TR 42/3000 ms; a rapid acquisition with relaxation enhancement factor of 8; 2 averages; 256×128 matrix, reconstructed to a 256×256 matrix; FOV $4 \times 4 \text{ cm}^2$, and slice thickness of 1.2 mm.

The T_{10} of the tumors was measured in a central slice by rapid acquisition relaxation enhancement (RARE) spin echo sequence with RARE factor of 2, TE of 15 ms and a series of variable repetition times, TRs (300 ms to 5000 ms); 128×64 matrix, reconstructed to a 128×128 matrix; FOV 4×4 cm², and slice thickness of 1.2 mm. Average T_{10} values were calculated in the ROI of the tumor. The T_{10} of plasma samples (~0.5 ml) obtained from blood samples of mice centrifuged at room temperature for 10 min (10,000g), was measured in 5mm NMR tubes at 9.4 T, 37°C, using a standard inversion recovery pulse sequence.

Dynamic contrast enhanced (DCE) MRI experiments were conducted with the ER targeted contrast agents, EPTA-Gd, TPTA-Gd, PTA-Gd and GdDTPA (Schering, Berlin, Germany). The protocol consisted of recording four pre-contrast followed by consecutive post contrast T1-weighted, 3D gradient-echo (3D-GE) pulse sequence with TE/TR 2.5/15 ms; flip angle 40°. Interleaved axial and coronal images of this sequence were applied before and after a single bolus injection using a spatial resolution of 0.156×0.156×1.2 mm³ in the axial scans and 0.234×0.156×1 mm³ in the coronal scans, with 4 averages (acquisition time 1.5 min) for EPTA-Gd and TPTA-Gd DCE experiments and 2 averages (acquisition time 0.75 min) for PTA-Gd and GdDTPA experiments. The axial scans were recorded for monitoring signal intensity in the tumors, and the coronal scans were recorded for monitoring signal intensity in the muscle and descending aorta.

Each contrast agent was injected as a bolus into the tail vein of the mice. The dose of EPTA-Gd was 0.03 mmol/kg (n=4) or 0.075 mmol/kg (n=5), and of TPTA-Gd 0.075 mmol/kg (n=4). The doses of PTA-Gd (n=4) and GdDTPA (n=2) were 0.15 mmol/kg and 0.4 mmol/kg, respectively.

Theory and data analysis

The relaxivities of the water nuclei in a solution of a Gd³⁺ chelate are determined by the contribution of the water molecules directly coordinated to the paramagnetic Gd³⁺ and transmitted to the bulk by chemical exchange (inner-sphere exchange) and by protons of water molecules interacting through space by dipolar mechanism via hydrogen bonding to hydrophilic groups (second-sphere exchange) and/or via the translational diffusion of water molecules surrounding the paramagnetic chelate (outer-sphere relaxation). The contribution of the second and outer sphere molecules to the relaxivity is difficult to quantify and estimation of these contributions requires fitting globally spectroscopic data obtained from studies of ¹⁷O NMR relaxation, nuclear magnetic relaxation dispersion and electron spin resonance relaxation with a theoretical model (42, 43). In this paper the solution NMR studies focused on investigating the inner-sphere exchange using ¹⁷O NMR. Although it was suggested that second and outer sphere contributions can contribute significantly to the T1 relaxivity of the water protons (44), based on recent analyses for acyclic Gd³⁺ chelates (45, 46) and on studies of the contrast agent MS-325 (gadofosveset), which has a similar molecular weight and structure in comparison to our ER targeted contrast agents (47), we assumed that in both EPTA-Gd and TPTA-Gd the inner-sphere contribution also dominates the proton water relaxivity at high magnetic field.

¹⁷O T2 relaxation studies—The kinetic parameters of the exchange of the water coordinated to Gd³⁺ in EPTA-Gd or TPTA-Gd (inner-sphere coordinated water) and the bulk water were determined by temperature dependent studies of the changes in the T2 relaxation rates of the ¹⁷O water due to the presence of the paramagnetic contrast agents. The paramagnetic induced change in the T2 relaxation rate, assuming only first hydration sphere contribution (48), is given by the Swift and Connick equation (49):

$$\frac{1}{T_2} - \frac{1}{T_{20}} = \frac{q[Gd]}{[H_2O]} \left[\frac{1}{\tau_m} \frac{(1/\tau_m + 1/T_{2m})/T_{2m} + (\Delta\omega_m)^2}{(1/\tau_m + 1/T_{2m})^2 + (\Delta\omega_m)^2} \right] \quad [1]$$

where $1/T_2$ and $1/T_{20}$ are the T2 relaxation rates in the presence and absence of the paramagnetic molecules, respectively, q is the number of coordinated water molecules to the Gd³⁺ ion, ω_m is the chemical shift difference between Gd-bound water and bulk water ¹⁷O nuclei, $1/T_{2m}$ is the Gd-bound ¹⁷O T2 relaxation rate, and k_{ex} is the exchange rate (equal to the inverse of the life-time of the Gd bound water, $1/\tau_m$). The contribution of ω_m^2 in Gd³⁺ complexes is usually negligible compared to other terms in Eq.[1] yielding to the following equation for the change in the T2 relaxation rate of bulk H₂¹⁷O:

$$\frac{1}{T_2} - \frac{1}{T_{20}} = \frac{q[Gd]}{[H_2O]} \left[\frac{1}{T_{2m} + \tau_m} \right] \quad [2]$$

The Gd-bound ¹⁷O T2 relaxation rate, $1/T_{2m}$, is dominated by the scalar-hyperfine interaction of the Gd³⁺ and ¹⁷O, and following the Solomon–Blombergen-Morgan (SBM) equation (50, 51) can be given by:

$$\frac{1}{T_{2m}} \cong \frac{1}{T_2^{sc}} = \frac{S(S+1)}{3} \left(\frac{A}{\hbar} \right)^2 \tau_{s1} \quad [3a]$$

$$\frac{1}{\tau_{s1}} = \frac{1}{\tau_m} + \frac{1}{T_{1e}} \quad [3b]$$

where S is the Gd³⁺ spin quantum number ($S=7/2$), $A/\hbar$ is the scalar-hyperfine coupling constant due to the interaction of the Gd³⁺ electronic spin and the ¹⁷O nuclear spin, $1/\tau_{s1}$ is the correlation time of this interaction which is the sum of the water exchange rate ($1/\tau_m$) and the electron spin relaxation rate of Gd³⁺ ($1/T_{1e}$).

The temperature dependence of τ_m and T_{1e} is given by the Eyring equation:

$$\frac{1}{\tau_m} = k_{ex} = \frac{k_{ex}^{298} T}{298.15} \exp \left[\frac{\Delta H}{R} \left(\frac{1}{298.15} - \frac{1}{T} \right) \right] \quad [3c]$$

$$\frac{1}{T_{1e}} = \frac{1}{T_{1e}^{298}} \exp \left[\frac{\Delta E_{T_{1e}}}{R} \left(\frac{1}{T} - \frac{1}{298.15} \right) \right] \quad [3d]$$

where k_{ex}^{298} is the rate of exchange at 298°K, H is the activation enthalpy, E_{Tle} is the activation energy for $1/T_{le}$, and R is the gas constant.

The changes in $1/T_2 - 1/T_{20}$ with temperature were fitted to Eqs. [2] and [3] using a non-linear least-square Levenberg-Marquardt algorithm (Origin, version 6.1). For this fitting we used a scalar-hyperfine constant of $(-3.8 \times 10^{-6} \text{ rad/s})$ (48, 42). We also assumed $q=1$, however, for $q=2$, all results will be the same aside from 50% reduction in k_{ex}^{298} . The fitting yielded four kinetic parameters: $k_{ex}^{298} (\text{s}^{-1})$, $H (\text{kJ mol}^{-1})$, $1/T_{le}^{298} (\text{s}^{-1})$, and $E_{Tle} (\text{kJ mol}^{-1})$ and estimated the goodness of the fitting by calculating a coefficient of determination, R^2 .

¹H T2 relaxation studies in H₂¹⁷O solutions—The ¹H T2 relaxation rate of free water enriched with 5% H₂¹⁷O was enhanced due to the spin-spin hyperfine interaction between ¹⁷O nuclei and the ¹H nuclei. In 5% H₂¹⁷O solutions of EPTA-Gd and TPTA-Gd the T2 relaxation rate of these protons reached values close to the inner-sphere exchange rates, enabling estimation of the water protons exchange rate through temperature dependent T2 relaxation studies and the SBM theory (50, 51). Since for the protons of water bound to Gd³⁺ the dipolar rather than the scalar mechanism dominates the T2 relaxation (52,53) the contribution of the scalar mechanism was neglected. We also applied the following two assumptions: 1. $\omega_S \gg \omega_I$, where ω_S and ω_I are the electronic and proton larmor frequencies respectively, and 2. the correlation time of the dipolar interaction, τ_c , is dominated by the rotational correlation time of the molecules and obeys $\omega_I^2 \tau_c^2 < 1$, as is the case for a similar Gd³⁺ contrast agent (47). The Gd-bound ¹H T₂ relaxation rate was therefore approximated to:

$$\frac{1}{T_{2m}} = \frac{1}{T_{2dd}} = \frac{\gamma_I^2 g^2 \mu_B^2 S(S+1)}{15r^6} \left[\frac{3\tau_{C1}}{1 + \omega_I^2 \tau_{C1}^2} + \frac{13\tau_{C2}}{1 + \omega_S^2 \tau_{C2}^2} + 4\tau_{C1} \right] \sim \frac{2.583 \times 10^{17}}{r^6} 7\tau_r \quad [4]$$

The distance of the water proton to Gd³⁺, r , was estimated to be 3.1 Å (54) and the temperature dependence of τ_r was assumed to follow the Eyring relation:

$$\tau_r = \tau_r^{298} \exp \left[\frac{E_r}{R} \left(\frac{1}{T} - \frac{1}{298} \right) \right] \quad [5]$$

where τ_r^{298} is the rotational correlation time at 298K and E_r is the activation energy for rotation. The observed changes in the T2 relaxation rates of ¹H nuclei with temperature were fitted to Eq.[2], incorporating Eq. [3c], the approximation in Eq.[4] and Eq. [5], using a non-linear least-square Levenberg-Marquardt algorithm (Origin, version 6.1). The fitting yielded four kinetic parameters: $k_{ex}^{298} (\text{s}^{-1})$, $H (\text{kJ mol}^{-1})$, $\tau_r^{298} (\text{s})$, and $E_r (\text{kJ mol}^{-1})$ and a coefficient of determination, R^2 .

In Vivo DCE-MRI—Signal intensities per pixel were normalized to the pre contrast intensity, yielding enhancement datasets defined as $(I(t) - I(0))/I(0)$, where $I(0)$ and $I(t)$ are the signal intensities pre- and at time t post-contrast, respectively. $I(0)$ per pixel was calculated as a mean intensity of the four pre-contrast images. Enhancements were compared for specific times post contrast injections.

Analysis of the entire DCE time course, over 60 min, was achieved by applying a model free method based on principal component analysis (PCA) algorithm as previously described (36, 55). Enhancement datasets of EPTA-Gd, TPTA-Gd and PTA-Gd were derived from one precontrast and 8 postcontrast images. The output yielded eight eigenvectors and eight projection coefficient maps, indexed and sorted according to their corresponding eigenvalues. The values of the projection coefficients of the 1st eigenvector depicted the overall extent of enhancement in the first 60 minutes providing a quantitative measure for comparing ER-positive and ER-negative tumors

Analysis of the dynamic contrast-enhanced datasets using a modified Tofts' kinetic model (56) was performed only for PTA-Gd and GdDTPA, introducing a disparity between the influx (K^{in}) and outflux (K^{out}) transcapillary transfer constants for the changes in the concentration of the contrast agent according to:

$$Ct(t) = DK^{in} \sum_{i=1}^2 \frac{a_i (e^{-(K^{out}/Ve)t} - e^{-m_i t})}{m_i - (K^{out}/Ve)} \quad [6]$$

Where D is the dose; Ve is the extravascular extracellular volume fraction set to 0.2 as previously measured in our laboratory for these tumors (57). $Ct(t)$ is the tissue concentration of the contrast agent and the plasma pharmacokinetic parameters, a_i , m_i ($i=1, 2$) (56). The pharmacokinetic parameters were determined from PTA-Gd and GdDTPA enhancement curves in the descending aorta by fitting the concentration evolution in the plasma, $Cp(t)$, to a bi-exponential decay: $Cp(t) = D (a_1 e^{-m_1 t} + a_2 e^{-m_2 t})$ using hematocrit of 0.4 (58), and plasma $T_{10} = 1824$ ms, measured separately in external plasma samples at 9.4T.

The relation between the enhancement and $Ct(t)$ (or $Cp(t)$) for a T1 weighted gradient echo sequence is given by :

$$\frac{I(t) - I(0)}{I(0)} = \frac{(1 - e^{-TR [\frac{1}{T_{10}} + r_1 Ct(t)]}) / (1 - \cos \alpha e^{-TR [\frac{1}{T_{10}} + r_1 Ct(t)]})}{(1 - e^{-\frac{TR}{T_{10}}}) / (1 - \cos \alpha e^{-\frac{TR}{T_{10}}})} - 1 \quad [7]$$

With r_1 of PTA-Gd $3 \text{ mM}^{-1}\text{s}^{-1}$ and of GdDTPA $4.3 \text{ mM}^{-1}\text{s}^{-1}$ (59). The T_{10} values of the tumors were measured separately as described above. Median $Ct(t)$ of tumors' ROI in a central slice were fitted to the model by nonlinear least square Levenberg–Marquardt algorithm (Origin, version 6.1).

Statistics

For data of cells in wells, averaged T1 relaxation times, $avgT_1$, were calculated from ~120 pixels of ROI in each well. The difference in $1/avgT_1$ of contrast agent bound cells and in contrast agent free cells, RI , served to compare between ER-positive and ER-negative cells using student's two-tailed paired t-test (GraphPad Software, Inc., QuickCalcs web site <http://www.graphpad.com/quickcalcs/ttest1.cfm>) to evaluate statistical significance.

In the *in vivo* studies, tumors' ROIs were delineated on the anatomical T2-weighted images and transferred to the corresponding DCE images. Pixel-by-pixel analysis of enhancement was performed in the whole tumor's ROI consisting of about several thousand pixels, depending on the tumor's size. Median enhancement values for each tumor and for muscle tissue were analyzed statistically for differences in enhancement using student's two-tailed paired t-test as described above. Median values of the projection coefficients per tumor and muscle tissue were calculated from pixel-by-pixel PCA of a central slice consisting of about several hundreds of pixels. Median kinetic parameters of PTA-Gd and GdDTPA perfusion were obtained from the fitting to the modified Tofts model of pixels in a central tumor slice that exhibited a coefficient of determination (also termed correlation coefficient value) $R^2 > 0.5$. Statistical differences of these medians were evaluated as described above for the enhancement. $P < 0.05$ were considered significant.

Results

^1H water Relaxivities in solution

The *r1* water relaxivities of the ER targeted contrast agents in PBS solutions (9.4T, 15°C) were $r1_{\text{EPTA-Gd}} = 6.8 \pm 0.7 \text{ mM}^{-1}\text{s}^{-1}$, and $r1_{\text{TPTA-Gd}} = 4.7 \pm 0.1 \text{ mM}^{-1}\text{s}^{-1}$. The *r1* of PTA-Gd measured under the same conditions was $r1_{\text{PTA-Gd}} = 3.0 \pm 0.1 \text{ mM}^{-1}\text{s}^{-1}$. EPTA-Gd demonstrated the highest efficiency as a T1 relaxation agent whereas PTA-Gd had the lowest *r1* relaxivity, most likely reflecting their difference in size.

Kinetics of water exchange

Water exchange between bulk water and EPTA-Gd or TPTA-Gd was investigated by measuring the ^{17}O T2 relaxation rate of H_2^{17}O as a function of temperature. The difference between the measured relaxation rate in a solution of each contrast agent, and of the contrast-free solutions ($1/T_2 - 1/T_{20}$) were measured at two concentrations of the contrast agent and were plotted as a function of temperature (Figure 1 B and C). The pattern of the curves indicated fast water exchange at high temperatures that approached an intermediate exchange rate and eventually reached the slow exchange limit at low temperatures. The curves were fitted to Eqs. [2] and [3], yielding four kinetic parameters, the ^{17}O water exchange rate at 298K, the activation enthalpy of the exchange, the electronic T1 relaxation rate at 298K, and the corresponding energy. The fitting was performed assuming $q=1$, based on the relaxivity of PTA-Gd of $3.0 \pm 0.1 \text{ mM}^{-1}\text{s}^{-1}$, which is slightly lower than the T1 relaxivities reported for similar contrast agents with $q=1$ (43). However, we cannot exclude the possibility that $q=2$, and consequently a twofold reduction in the exchange rates (Table 1). Similar exchange kinetic parameters were obtained for the two concentrations of EPTA-Gd. Although the enthalpy of activation of TPTA-Gd exchange appeared to be approximately 30% higher at 1mM than in 2.5mM, within the error of their fitting these values were of the same order of magnitude (Table 1). We also measured the temperature dependent T2 relaxation rates of water protons in the 5% H_2^{17}O solutions of EPTA-Gd and TPTA-Gd. In these solutions the water proton T2 relaxation rate was enhanced by the ^1H - ^{17}O spin-spin scalar interaction reaching rates close to the water exchange rates. The temperature depended changes in the T2 relaxation rates (Figure 1 D and E) indicated reaching intermediate and slow exchange rates at temperatures below 290K enabling

estimation of the rate of water protons' exchange and the activation enthalpy, as well as the rotational correlation time and the activation energy (Table 2).

Biological activity and contrast enhancement in human breast cancer cells

Evaluation of the hormonal activities induced by EPTA-Gd and TPTA-Gd was performed in estrogen responsive MCF7 and T47D human breast cancer cells. Both agents added at micromolar concentrations enhanced MCF7 and T47D cell proliferation rate in reference to the proliferation in hormone free medium and similar to the enhancement induced in by the native estrogen added in nanomolar concentration. EPTA-Gd (0.1–2 μ M) stimulated the proliferation of the cells in a dose dependent manner augmenting by twofold the proliferation rate ($P < 0.02$, $n = 5$); TPTA-Gd, (0.1–10 μ M) stimulated cell proliferation to 1.7 fold ($P < 0.01$, $n = 7$). Both agents added at a concentration of 5 μ M upregulated c-Myc expression level within 1–2 h, EPTA-Gd by 250% and TPTA-Gd by 150%. They also reduced at this concentration ER expression level, EPTA-Gd to 25% and TPTA-Gd to 60% of the initial level. These specific activities reflect induction of the transcription pathways in the nuclei via binding to nuclear ER in a similar manner to the activation by 17 β -estradiol (40).

T1 relaxation measurements were conducted in ER-transfected (ER-positive) and ER-negative MDA-MB-231 human breast cancer cells. The ER-negative cells served to determine changes due to non-specific binding and possible decomposition of the contrast agents inside the cells. The difference in the relaxation rates between the two cell systems served, therefore, to reveal the sole effect due to binding to the receptor. The experiments were performed in two experimental set-ups: Cells cultivated on beads ($\sim 4 \times 10^7$ cells/1ml beads) were either suspended in wells (23°C) or perfused in NMR tubes (36°C).

T1 relaxation rates were determined in cells in wells that were incubated, and suspended in the wells with contrast free medium (contrast agent free cells were termed control samples) and cells that were incubated with each targeted contrast agent and underwent washout of the medium which removed the non-bound extracellular contrast agent, yielding cells with bound intracellular contrast agent (termed contrast agent bound cells). Experiments showing T1 maps of contrast agent free (control) and contrast agent bound ER-positive and ER-negative cells are demonstrated in Figure 2. In this experiment the difference in the T1 relaxation rates of contrast agent bound cells and contrast agent free cells, RI , were higher in ER-positive than in ER-negative cells. Statistical analysis of repetitive experiments showed that EPTA-Gd induced consistently higher RI values in ER-positive cells as compared to the ER-negative cells ($P = 0.03$, $n = 9$, Figure 2C). However, for TPTA-Gd, RI showed a trend of higher values in ER-positive cells but this trend was not significantly different between ER-positive and negative cells ($P = 0.13$, $n = 8$, Figure 2C).

In studies of perfused cells, concentration dependent changes in T1 relaxation rates in the ER positive and ER-negative cells, placed at the same density and amount in the perfused NMR tube, were measured before and after administrating increased amounts of the contrast agent into the perfusion medium (Figure 3 and Figure S1 and Tables S1 and S2). ^{31}P NMR spectra recorded at the beginning and at the end of the experiments indicated constant ATP levels and validated cell viability throughout the experiments (Figure 3A). The measured

EPTA-Gd and TPTA-Gd concentration dependent changes in the T1 relaxation rate served to calculate a parameter which reflected the changes in the effective T1 relaxivity of the water inside and outside the cells. This concentration dependent change was higher in the ER-positive cells' system than that in the ER-negative cells' system (Figure 3 B and C, Table S2). The consistent higher relaxivity in ER-positive cells, which aside from ER were the same as the ER-negative cells, indicated that interaction with ER is responsible for the difference in the concentration dependent changes. For both agents these concentration dependent changes in the ER-negative cells' system were higher than those determined in solution, possibly reflecting interaction with extracellular and or intra-cellular components as well as in the viscosity in the two compartments. The relatively high concentration dependent changes in the T1 relaxation of of TPTA-Gd as compared to EPTA-Gd in ER-negative cells suggested high non specific interactions of this agent with cellular components, other than ER. Overall, the changes in the EPTA-Gd and TPTA-Gd concentration dependent T1 relaxation rates in the two cell systems suggested a better efficiency of the former agent to induce contrast in ER-positive cells due to interaction with ER.

In vivo MRI of breast cancer xenografts

The in vivo DCE-MRI investigations of EPTA-Gd and TPTA-Gd were performed on ER-positive and ER-negative xenografts implanted orthotopically in the same mouse. Bolus injection of each contrast agent induced signal enhancement in the tumors reaching a maximum level at approximately 20 minutes post injection and maintaining the enhancement constant for at least 150 minutes post injection. The enhancement induced by EPTA-Gd at 20 minutes was significantly higher in the ER-positive tumors as compared to ER-negative tumors and muscle tissue (Figure 4A and Figure 5A). The enhancement induced by TPTA-Gd in ER-positive tumors was low and not significantly different between the ER-positive and negative tumors (Figure 4B and Figure 5A). An unexpected high enhancement was induced by TPTA-Gd in muscle tissues (Figure 4B and Figure 5A) demonstrating non ER specific interaction of this agent.

Further quantitative evaluation of the enhancement patterns of the dynamic experiment during the first 60 minutes, using principal component analysis (PCA), yielded eight eigenvectors arranged according to their eigenvalues, with the 1st and dominant eigenvector depicting the variance due to the enhancement caused by the various agents and the remaining eigenvectors with very low eigenvalues, depicting predominantly noise fluctuations (36). The projection coefficient parametric maps of the 1st eigenvector confirmed the enhancement results: EPTA-Gd injection yielded significantly higher projection coefficients values in the ER-positive tumors as compared to ER-negative tumors and muscle tissue (Figure 4C and Figure 5B). TPTA-Gd injection yielded low and similar projection coefficients in both tumor types whereas the muscle projection coefficient were high (Figure 4D and Figure 5B),

In order to examine whether differences in the perfusion capacity are responsible for the observed differences between ER-positive and negative tumors we applied DCE measurements using the extracellular contrast agents, PTA-Gd and GdDTPA. Figure 6

demonstrates representative time courses of these contrast agents measured in the plasma and in the tumors. The time courses were analyzed using a modified Toft's model, yielding kinetic parameters for the transcapillary influx and outflux rates. Statistical analysis of the kinetic parameters (Table 3) clearly showed a similar plasma kinetics and perfusion kinetics in the ER-positive and negative tumors for both contrast agents. Further analysis of the PTA-Gd enhancement at 20 minutes (Figure 5A), and of the PCA analysis of PTA-Gd time courses (Figure 5B) confirmed the similarity in the perfusion capacity of both tumors. Thus, the higher enhancement of EPTA-Gd in the ER-positive tumors reflects predominantly specific binding to ER.

Discussion

The estrogen receptor is predominantly localized inside cells, in the nucleus. The molecular weight of EPTA-Gd and TPTA-Gd, as well as their lipophilic targeting moieties made them good candidates for crossing cell and nuclear membranes. The conformation and flexibility of the binding cavity and surrounding environment of ER enabled binding of the targeting moiety and accommodation of the bulky PTA-Gd moiety (26, 60). In this article we describe the distinct magnetic resonance characteristics of these new ER targeted contrast agents in solution, in breast cancer cells and in breast cancer xenografts in mice.

Studies in solutions showed the ability of these two contrast agents to augment T1 relaxation rate of the water protons. The T1 relaxivity of EPTA-Gd and TPTA-Gd was higher than that of PTA-Gd. The correlation between molecular weight and proton T1 relaxivity per Gd^{3+} has been previously demonstrated by Tweedle and co-authors for a series of Gd^{3+} -chelates (61). This explains the higher relaxivity of these agents (with MW of ~900 Dalton) as compared to that of PTA-Gd (with MW of 563.5 Dalton).

In general, the bulk ^1H T1 relaxivities of water induced by Gd^{3+} -chelates depend on various physical and chemical properties, including the number of water molecules in the 1st coordination sphere and the outer coordination sphere, as well as the inner and outer sphere water exchange rate(s) and mechanism(s), and the field strength (43, 62–64). Using temperature dependent ^{17}O and ^1H T2 relaxation rate measurements of H_2^{17}O solutions of EPTA-Gd or TPTA-Gd we investigated the exchange of the water. The exchange rates calculated from temperature dependent studies of ^{17}O labeled water, assuming $q=2$, were in the range found for the common extracellular Gd-based contrast agents, [for GdDTPA $k_{ex}^{298}=(4.1 \pm 0.3) \times 10^6 \text{ s}^{-1}$ and for GdDOTA $k_{ex}^{298}=(4.8 \pm 0.4) \times 10^6$ (65)] whereas for $q=1$ a twofold faster exchange rates were calculated. These rates are by more than three orders of magnitude slower than the reported values for outer-sphere exchange rates (45). Based on previous studies with lanthanide chelates (62, 63), it appeared reasonable to suggest that for $q=1$, the transition state of the exchange process can easily accommodate another water molecule and occur by an associative fast mechanism. However, as we cannot rule out that two water molecules occupy the first coordination sphere of the ER targeted contrast agents, the exchange mechanism could be dissociative with a rate similar to that of the nine coordinated Gd^{3+} -chelates such as GdDTPA and GdDOTA chelates (62, 63). In addition to the uncertainty in the number of water molecules in the 1st coordination sphere, we have also neglected contribution due to zero field splitting (ZFS), as for Gd^{3+} at high fields the

ZFS is small compared to the Zeeman energy and hence to a first approximation negligible (64).

The enthalpy of activation of the exchange process was in a range reported for other low MW Gd³⁺-chelates (43, 66, 67). The difference between the enthalpy of activation for the two concentrations of TPTA-Gd, although within the error of fitting, may reflect partial aggregation at high temperatures, but further concentration and temperature dependent studies are required to find out a dimerization or other aggregation process of these contrast agents.

The electronic relaxation rates of Gd³⁺ ion, were within the range reported previously for this ion, 10⁷–10⁹ s⁻¹ at ~298K (67–69). The electronic activation energies exhibited high standard error. Evaluation by serial fitting simulations showed that the fitting process was highly sensitive to changes in the exchange rate and enthalpy of the exchange but much less sensitive to changes in the electronic relaxation rate and activation energy, as was previously reported in studies of other paramagnetic contrast agents (65).

The exchange rates of the water protons measured H₂¹⁷O solutions indicated approximately twofold faster exchange rates than those of water ¹⁷O exchange rates (Table 2). In general, ¹⁷O exchange reflects a whole water molecule exchange whereas the proton exchange can be dominated by a protonation exchange process. The faster proton water exchange rate suggests contribution of a protonation exchange mechanism. However, further pH dependent studies need to be conducted to verify the mechanism, as the measurements were performed at a pH close to neutral and the difference between the whole water exchange and the protonation exchange appeared to be marginal.

We also calculated a rotational correlation time $\tau_r^{298} = 0.78$ ns and $\tau_r^{310} = 0.36$ ns (Table 2). These values were compared with the values calculated using the Stokes-Einstein-Debye equation and an effective radius of EPTA-Gd of 4.17 Å obtained from the volume and surface area determined by x-ray crystallography (60), using Voss and Gerstein methodology and software (70). This calculation yielded $\tau_r^{298} = 0.074$ ns and $\tau_r^{310} = 0.050$ ns. Calculation of τ_r using the long axis of EPTA-Gd, with a radius of 8.85 Å, yielded a rotational correlation time $\tau_r^{298} = 0.72$ ns and $\tau_r^{310} = 0.37$ ns and. Clearly, as the ER targeted agents are not spherical and the contribution of the hydration sphere was neglected, the above calculations provided only a rough estimation of the lower and upper limits of the rotational correlation time. The value calculated from the proton relaxation studies indicated that at 310K and higher temperatures, where T_{2m} dominated the relaxation of the water bound to Gd³⁺, all values of the rotational correlation time given above obeyed the assumption $\omega_I^2 \tau_c^2 < 1$ (see Eq. 5 in Theory and data analysis). At lower temperatures this assumption was not justified as $\omega_I^2 \tau_c^2 > 1$, however, at these temperatures τ_m , rather than T_{2m}, started to dominate the T2 relaxation rate difference. Interestingly, studies of the contrast agent MS-325, which is similar in MW and structure to our ER targeted contrast agents, yielded $\tau_r^{310} = 0.115$ ns (47). In order to obtain a precise estimation of the rotational correlation time additional studies, including ¹H nuclear magnetic relaxation dispersion, are necessary.

In order to characterize the T1 induced enhancement upon binding of the contrast agents to ER we genetically engineered human breast cancer cells to express high amounts of ER and used the wild type cells of the same origin as control cells. This enabled us to isolate the interactions and enhancement due to binding to ER alone with no need to account for differences between different cell types. Upon binding of the ER targeted contrast agents to the receptor ($ER_{MW}=66.4$ kDa) the rotational correlation time increases. Both EPTA-Gd and TPTA-Gd do not bind covalently to ER and their rotation may have mobility freedom with a shorter correlation time than the entire protein. The binding may also affect the hydration number and water exchange rate and mechanism, thereby modulate the relaxivity to values different from those expected from the free contrast agent relaxivity (71) Indeed at high fields increasing the rotational correlation time may reduce rather than increase the relaxivity. Nevertheless, in specific cases, such as with the contrast agent MS-325 (Gadofosveset), which is close in MW and structure to our contrast agents and binds albumin non covalently, it was shown that even at 9.4 T the relaxivity of the free agent ($5.14 \text{ mM}^{-1}\text{s}^{-1}$) is less than the relaxivity when bound to human serum albumin ($7.16 \text{ mM}^{-1}\text{s}^{-1}$) (72). Thus, at high fields the T1 relaxivity of the ER-targeted contrast agents in the cells may increase or decrease due to interaction with cellular components (73–75). The concentration dependent studies of perfused cells suggested an increase in the T1 proton relaxation rate upon interaction with cells, particularly ER-positive cells, although the exact increase is not known. Binding affinity studies with the purified estrogen receptor in solution (36) showed that despite the bulky organometallic moiety at the 17α -position of 17β -estradiol or tamoxifen, these contrast agents interact with ER with a relatively high affinity, in the micromolar range. The mode of binding was also demonstrated by elucidating the crystallographic structure of EPTA-Eu bound to the ligand binding domain of ER. This structure shows that EPTA-Gd forms a complex with ER, maintaining the conformation in the binding cavity similar to that of 17β -estradiol and extending the paramagnetic moiety towards the protein water interface (60).

The ability of the two targeted contrast agents to act as selective estrogen receptor modulators through binding to the nuclear receptor was verified by measuring specific cellular activities such as cell proliferation, induction of c-Myc expression level and reduction of ER expression level (40). Both agents were found to mimic these estrogen induced changes acting as agonists, with EPTA-Gd showing a stronger agonistic activity than TPTA-Gd. These results served to show that indeed these agents entered the nuclei and interacted with ER in the same manner as 17β -estradiol inducing the signaling pathways typical to this hormonal regulation.

The T1 relaxation rates in ER positive and ER-negative cells incubated in wells further validated specific binding to ER (Figure 2). The results clearly showed significant T1 augmentation by EPTA-Gd in ER-positive cells as compared to ER-negative cells, however, T1 relaxation augmentation by TPTA-Gd was not significant, suggesting that strong non specific binding to intracellular components may mask the specific binding to ER. Relaxation measurements using a cell perfusion system and varying the concentration of the contrast agent for the same cell system confirmed the results obtained in the wells: The concentration dependent change of the T1 relaxation rate induced by EPTA-Gd in this system was 4–5 times higher than in solution and 45% higher in ER-positive as compared to

ER-negative cells. The concentration dependent change in T1 relaxation induced by TPTA-Gd was approximately twice higher than that of EPTA-Gd but the difference between ER-positive and negative cells was low (22%), indicating high non ER-specific enhancement, most likely due to interaction of the tamoxifen moiety with other cellular components. Overall, the relaxation measurements of EPTA-Gd in the cells' systems enabled separation of specific binding to ER from non-specific interactions with other cellular components. This separation was less pronounced for TPTA-Gd which exhibited strong non-specific binding to unknown cellular components.

The in vivo efficiency of EPTA-Gd to interact with ER and enhance the T1 relaxation rate confirmed the results obtained with EPTA-Gd in cell cultures. Monitoring the ER-positive and ER-negative breast cancer xenografts in the same mouse ensured the same arterial input function and hence differences resulting from interaction within the xenografts themselves. To further ensure that the specific enhancement of the ER-positive xenografts is not due to a better blood perfusion of these tumors, we performed DCE-MRI measurements using the extracellular contrast agents, PTA-Gd and GdDTPA. The kinetic parameters obtained by model based analysis demonstrated for both extracellular agents similar kinetic parameters and hence similar perfusion efficiency of the ER-positive and negative xenografts.

The in vivo studies uniquely showed an unexpected accumulation of TPTA-Gd in muscle tissue. Although a statistical borderline significance ($P < 0.055$) was evaluated for the difference between ER-positive tumors and muscle tissue, the trend of high enhancement in the muscle was clear (Figure 4B, and 5A). This accumulation could not be inferred from the cell culture studies, although high non specific binding to cellular components was also found in the cells. Since the fraction of muscle weight in the mouse is high, most of the contrast agent accumulated in this tissue, reducing the effective dose that reached the tumors to low levels, insufficient for significant ER binding.

It is reasonable to suggest that structural differences between the estradiol and tamoxifen derivatives led to the observed differences in the interaction in vivo. The rigidity of the targeting moiety of EPTA-Gd and its similar interaction and structure to 17β -estradiol when bound to ER appeared to determine its ability to serve as a targeted ER probe. In contrast, the flexibility of the tamoxifen moiety appeared to induce non specific binding to other cellular components, not only in breast cancer cells but also in muscle tissue, and limited its ability to serve as a targeted ER probe in vivo.

In summary, we have characterized the solution, cellular and in vivo properties of two new contrast agents targeted to the estrogen receptor. In solution the magnetic resonance characteristics of both targeted contrast agents and their binding affinity to ER were similar. However, a disparity in their ability to interact with other cellular components was discovered in the cellular studies and particularly in the in vivo studies. Overall, we have shown that the NMR characteristics in solution, cells and tumors in vivo lay a basis for the molecular imaging of the estrogen receptor using EPTA-Gd as a template for further developments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. ¹⁷O T₂ relaxation measurements in H₂¹⁷O (5% enriched) PBS solutions of EPTA-Gd and TPTA-Gd

A. The chemical structure of 17 β -estradiol pyridine-tetra-acetate-Gd (EPTA-Gd) and tamoxifen pyridine-tetra-acetate-Gd (TPTA-Gd), and their molecular weights.

B,C. Temperature dependence of the paramagnetic contribution to ¹⁷O T₂ relaxation rate due to the presence of 1 mM (squares) or of 2.5 mM (circles) EPTA-Gd (B) or TPTA-Gd (C).

D,E. Temperature dependence of the paramagnetic contribution to ^1H T2 relaxation of H_2^{17}O PBS solution of 2.5 mM EPTA-Gd as in B (D) and 1 mM TPTA-Gd as in C (E). Solid lines represent least square fits of the experimental data points as described in "Theory and data analysis".

Figure 2. T1 relaxation induced by EPTA-Gd and TPTA-Gd in ER-positive and ER-negative breast cancer cells

A,B. T1 maps of ER-positive and ER-negative MDA-MB-231 human breast cancer cells. The cells in the wells were cultivated on Biosilon polystyrene beads. Before the T1 measurements control samples were incubated with medium free of contrast agent for 60 min at 37°C and then transferred to the wells in contrast agent free medium. Contrast agent bound cells were obtained after washing out the contrast agent from the medium. These samples were incubated for 60 min at 37°C in medium containing 6 μM EPTA-Gd (A) or 5 μM TPTA-Gd (B) and then were washed with contrast agent free medium and transferred to the wells in the contrast agent free medium. Relaxation rates were determined at 23°C at 4.7T.

C. Statistical analysis of the change in relaxation rate, R_1 , of repetitive experiments with EPTA-Gd or TPTA-Gd. n - number of experiments.

Figure 3. Concentration dependent changes in the T1 relaxation rate of EPTA-Gd and TPTA-Gd in ER-positive and ER-negative cells perfused at $36 \pm 1^\circ\text{C}$

The cells were perfused in the NMR tube at $36 \pm 1^\circ\text{C}$ and treated with increased concentrations of each contrast agent. T1 relaxation rates of water protons were determined using inversion recovery pulse sequence at 9.4 T. The concentration dependent changes in the differences in the relaxation rates in the presence and absence of each contrast agent were calculated as described in Methods. The values of the measured T1 relaxation times $\pm$ SD are summarized in a supplementary Table S1.

A. ³¹P NMR spectra of perfused ER-positive and ER-negative MDA-MB-231 human breast cancer cells at the start and the end of an experiment. PCho, phosphocholine; Pi, inorganic phosphate; NTP, nucleoside triphosphate.

B. Change in T1 relaxation rate with increased concentration of EPTA-Gd in perfused ER-positive and ER-negative cells. The concentration dependent changes in the relaxation rates are linear yielding the following slopes: $r_{\text{EPTA-Gd}}(\text{ER-positive}) = 28.5 \pm 0.1 \text{ mM}^{-1} \text{ s}^{-1}$ ($n=2$) and $r_{\text{EPTA-Gd}}(\text{ER-negative}) = 19.6 \text{ mM}^{-1} \text{ s}^{-1}$. The standard deviations of R_1 are presented in a supplementary Table S2.

C. Changes in T1 relaxation rate with increased concentration of TPTA-Gd in perfused ER-positive and ER-negative cells. The concentration dependent changes in the relaxation rates

are linear yielding the following slopes: $rI_{TPTA-Gd}(ER-positive) = 42.1 \text{ mM}^{-1} \text{ s}^{-1}$, and $rI_{TPTA-Gd}(ER-negative) = 36 \text{ mM}^{-1} \text{ s}^{-1}$. The standard deviations of $R1$ are presented in a supplementary Table S2.

Figure 4. EPTA-Gd and TPTA-Gd DCE-MRI in ER-positive and ER-negative breast cancer xenografts in mice

A,B. Enhancement maps of ER-positive and ER-negative human MDA-MB-231 breast tumors (left, axial scan) and of muscle tissue (right, coronal scan), overlaid on their corresponding T1-weighted images, 20 minutes post bolus injection to the tail vein of 0.075 mmol/kg EPTA-Gd (A) or TPTA-Gd (B).

C,D. Projection coefficient maps of the 1st eigenvector in the tumors' ROI (left, axial scan) and of a muscle tissue (right, coronal scan) overlaid on the T1-weighted images.

Figure 5. Statistics of enhancement and 1st projection coefficient derived from DCE-MRI with EPTA-Gd, TPTA-Gd, and PTA-Gd

A. Enhancement at 20 minutes post contrast in ER-positive and ER-negative tumors and of muscle tissue. Data presented as mean $\pm$ SEM of median enhancement values calculated from all pixels in the ROIs of the slices of each tumor.

B. Projection coefficient (pc) values of the first eigenvector (EV) in ER-positive and ER-negative tumors and of muscle tissue. Data presented as mean $\pm$ SEM of pc values, calculated as described in Methods.

P values were calculated by student's two-tailed paired t-test, n - number of experiments.

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Figure 6. Typical time courses of PTA-Gd and GdDTPA in the plasma and in ER-positive and ER-negative tumors

Change in the concentration of the contrast agent in the plasma (C_p) or in the tumors (C_t) after bolus injection of 0.15 mmol/kg PTA-Gd (A,B) or 0.4 mmol/kg GdDTPA (C,D) into the tail vein of the mice.

Enhancement in plasma were derived from ROI in the descending aorta and the concentration time course in plasma was fitted to a bi-exponential decay to yield pharmacokinetics rate constants using a plasma $T_{10} = 1824$ ms, measured separately in

external plasma samples at 9.4T, as described in Methods. Data in tumors were obtained from tumor pixels in a central slice that were fitted to the modified Tofts kinetic model as described in Methods. Average values of the pharmacokinetic parameters and transcapillary transfer constants are summarized in Table 3.

Table 1

Water exchange and electronic relaxation parameters of EPTA-Gd and TPTA-Gd in PBS solutions.

Exchange parameters ^a					
	$k_{ex}^{298} (s^{-1})^b$	H (kJ mol ⁻¹)	1/T _{1e} ²⁹⁸ (×10 ⁷ s ⁻¹)	ET1e (kJ mol ⁻¹)	R ²
EPTA-Gd	1 mM (1.0 ± 0.2) × 10 ⁷	56.4 ± 7.2	1.16 ± 0.56	57.1 ± 110	0.97
	2.5 mM (1.1 ± 0.3) × 10 ⁷	61.4 ± 7.7	1.0 ± 1.4	29.3 ± 332	0.99
TPTA-Gd	1 mM (0.9 ± 0.4) × 10 ⁷	69.5 ± 15	1.67 ± 0.12	-51.6 ± 35	0.96
	2.5 mM (0.8 ± 0.3) × 10 ⁷	48.7 ± 6.7	1.76 ± 0.12	-9.7 ± 29	0.95

^a The exchange parameters are defined in Methods.

^b For the calculation of k_{ex}^{298} the inner sphere water coordination number was q=1. For q=2 the k_{ex}^{298} need to be divided by 2, all other parameters remain the same.

Table 2

Proton water exchange and electronic relaxation parameters of EPTA-Gd and TPTA-Gd in PBS solutions.

		Exchange Parameters ^d				R ²
		k_{ex}^{298} (s ⁻¹) ^b	H (kJ mol ⁻¹)	τ_r^{298} (×10 ⁻⁹ s)	E _r (kJ mol ⁻¹)	
EPTA-Gd	2.5mM	(3.0±2.2)×10 ⁷	77.5 ± 23.4	0.78 ± 0.05	47.3 ± 5	0.98
TPTA-Gd	1mM	(1.4 ± 0.1)×10 ⁷	51.4 ± 2.3	1.3 ± 0.3	63.8 ± 2.1	0.99

^aThe parameters are defined in Methods.

^bFor the calculation of k_{ex}^{298} the inner sphere water coordination number was q=1. For q=2 the k_{ex}^{298} need to be divided by 2, all other parameters remain the same.

Table 3

PTA-Gd and GdDTPA kinetic parameters in the plasma of SCID mice and ER-Positive and negative breast cancer xenografts.

	PTA-Gd (n=4) ^c		GdDTPA (n=2) ^c	
D (mmol/kg)	0.15		0.4	
Plasma				
a1 (kg/l) ^a	20±12.6		4.0±0.2	
a2 (kg/l) ^a	3.8±1.8		4.5±0.1	
m1 (min ⁻¹) ^a	0.48±0.16		0.21±0.17	
m2 (min ⁻¹) ^a	0.04±0.02		0.02±0.03	
Tumors	ER-positive	ER-negative	ER-positive	ER-negative
K^{in} (min ⁻¹) ^b	$(2.5\pm0.6) \times 10^{-2}$	$(2.7\pm0.9) \times 10^{-2}$	$(1.9\pm0.7) \times 10^{-2}$	$(1.6\pm0.2) \times 10^{-2}$
	P= 0.64 ^d		P=0.58 ^d	
K^{out} (min ⁻¹) ^b	$(2.5\pm1.1) \times 10^{-2}$	$(2.6\pm0.6)\times 10^{-2}$	$(2.0\pm0.04) \times 10^{-2}$	$(2.0\pm0.1)\times 10^{-2}$
	P=0.74 ^d		P=0.5 ^d	

^a (a1,a2) and (m1,m2) are the decay amplitude and time constants respectively in the plasma defined in Methods

^b (K^{in}) and (K^{out}) are the influx and outflux transcapillary transfer constants defined in Methods

^c n indicates number of experiments.

^d P values obtained using a two-tailed paired t-test,