In Vivo Magnetic Resonance Imaging of the Estrogen Receptor in an Orthotopic Model of Human Breast Cancer

Adi Pais¹, Chidambaram Gunanathan², Raanan Margalit¹, Inbal Eti Biton³, Ady Yosepovich⁴,⁵, David Milstein², and Hadassa Degani¹

Abstract

Histologic overexpression of the estrogen receptor α (ER) is a well-established prognostic marker in breast cancer. Noninvasive imaging techniques that could detect ER overexpression would be useful in a variety of settings where patients’ biopsies are problematic to obtain. This study focused on developing, by in vivo MRI, strategies to measure the level of ER expression in an orthotopic mouse model of human breast cancer. Specifically, novel ER-targeted contrast agents based on pyridine-tetra-acetate-Gd(III) chelate (PTA-Gd) conjugated to 17β-estradiol (EPTA-Gd) or to tamoxifen (TPTA-Gd) were examined in ER-positive or ER-negative tumors. Detection of specific interactions of PTA-Gd with ER were documented that could differentiate ER-positive and ER-negative tumors. In vivo competition experiments confirmed that the enhanced detection capability of EPTA-Gd was based specifically on ER targeting. In contrast, PTA-Gd acted as an extracellular probe that enhanced ER detection similarly in either tumor type, confirming a similar vascular perfusion efficiency in ER-positive and ER-negative tumors in the model. Finally, TPTA-Gd accumulated selectively in muscle and could not preferentially identify ER-positive tumors. Together, these results define a novel MRI probe that can permit selective noninvasive imaging of ER-positive tumors in vivo. Cancer Res; 71(24); 7387–97. ©2011 AACR.

Introduction

During the last decades, intensive efforts have been devoted to improve the management of breast cancer, the most common malignancy in women. These efforts led to the discovery of novel means of diagnosis (1), as well as of specific biomarkers for classifying molecular subtypes and assessing prognosis (2–7). Currently the predominant prognostic biomarkers of breast cancer are the receptors to estrogen and progesterone (8–11), human epidermal growth factor receptors HER2/ErbB2 and HER1 (12–14), and various cytokeratins (15, 16). The presence of high level of the estrogen receptor (ER) serves also to select patients for hormonal therapy.

Estrogen receptor is a member of the nuclear receptor family discovered 50 years ago (17). It is a key regulator in female reproductive organs, in the mammary gland, as well as in the development and function of the skeletal and cardiovascular systems (18). Estrogen (E2) enters freely the cells, binds to the estrogen receptors, ERα and ERβ, which mediate estrogen signaling by activation or repression of gene transcription (19). In breast cancer, the two ERs have distinct expression patterns, with higher ERα and lower ERβ levels observed in breast cancer cells than in normal mammary epithelial cells (20). By inducing cell proliferation (21), the ERα contributes significantly to the growth and progression of E2-dependent breast tumors.

The current clinical methods for assessing the level of ER, such as enzyme immunoassay or immunohistochemistry, suffer from analytic variations and lack of standardization across laboratories (22–24). In the last 2 decades, noninvasive in vivo imaging modalities such as PET (positron emission tomography) and SPECT (single-photon emission computed tomography) have attempted to image and determine ER level in patients with breast cancer by using radiolabeled ER ligands. PET and SPECT tracers such as ¹⁹⁸Tc and ¹²⁴I-estrogen analogous have been tested in early clinical studies (25). New estrogen-derived steroidal diamagnetic metal complexes (26), as well as ⁹⁹Tc/Re chelates (27), targeted to ER were tested in cell cultures in vitro.

Recently, we have described a paramagnetic MRI probe designed to be targeted to the ER, based on 17β-estradiol conjugated to a Gd⁴⁺ chelate of pyridine-tetra-acetate–EPTA-Gd ([estradiol-17R-yethyl]n[pyrdin-2,6diyl]n(his[methylene-nitrilo]) tetraakis(acetate)] discussed in the work of Gunanathan and colleagues (28), and determined the X-ray structure of the europium chelate of EPTA-Gd bound to the ligand-binding domain of ERα (29). MRI probes targeted to the progesterone receptor were also described (30, 31). In this study, we present for the first time in vivo MRI application of the EPTA-Gd probe and of another novel probe based on tamoxifen conjugated to pyridine-tetra-acetate-Gd (TPTA-Gd) in a mouse...
model of human breast cancer. As a control, we have applied a new nontargeted contrast agent composed of the paramagnetic center of the above two targeted probes, pyridine-tetra-acetate-Gd (PTA-Gd). The time courses of contrast enhancement induced by each probe were monitored simultaneously in ER-transfected (ER-positive) and in wild-type (ER-negative) MDA-MB-231 human breast cancer tumors, implanted orthotopically in the same mouse. The results showed specific in vivo interaction of EPTA-Gd with ER, differentiating between ER-positive and ER-negative tumors, whereas TPTA-Gd appeared to bind more selectively to muscle tissues and did not show preferential in vivo interaction with ER.

Materials and Methods

Contrast agents synthesis

Two novel contrast agents targeted to the ER, based on the conjugation of PTA-Gd chelate to the native ligand, 17β-estradiol, (EPTA-Gd) or to the anticancer drug, tamoxifen, (TPTA-Gd), were synthesized (Fig. 1). The synthesis of PTA-Gd (32) and EPTA-Gd (28) were previously reported. The synthesis of TPTA-Gd is described in the Supplementary Data and Figs. S1–S4.

Binding to ER and relaxivity in solution

The binding affinities were measured by a radioactive inhibitory competitive assay in a cell-free system, using recombinant hERα (1.76 nmol/L; PanVera Inc.), tritiated 17β-estradiol that would be bound to the hERα, and ER-positive and ER-negative tumors. Experiments were carried out in duplicates. The IC50 values (the concentrations of competing ligand required to replace half of the tritiated estradiol of 0.2 nmol/L (34)). The IC50 values were then converted to absolute inhibition constants Ki as defined as the concentration of competing ligand which binds half of the receptors when no ligands are present, using the Cheng–Prusoff equation (33) and using a Kd of 17β-estradiol of 0.2 nmol/L (34).

T1 relaxation rates of water protons were measured as a function of the concentration of EPTA-Gd, TPTA-Gd, and PTA-Gd in phosphate buffer solutions at 9.4T (400 MHz) using either a spectroscopic inversion recovery pulse sequence or a spin echo imaging sequence with a constant echo time and a series of variable repetition times. The T1 relaxivity, r1, was calculated from the slope of the linear fit of 1/T1 − 1/T1(0) versus the concentration of each contrast agent, where 1/T1(0) is the relaxation rate in a contrast agent–free solution.

Cells

MDA-MB-231 human breast cancer cells were obtained from the American Type Culture Collection. Cells were routinely cultured under standard conditions as described previously (35). MDA-MB-231 with tetracycline-inducible ER were obtained by transfection with a plasmid encoding tetracycline repressor protein (TR) pcDNA6/TR (T-REx System; Invitrogen) and with a plasmid encoding ERα pcDNA4/ER (kindly provided Prof. Pierre Chambon, Université de Strasbourg, France) as described in the Supplementary Part. The ER expression level of ER-transfected MDA-MB-231 (ER-positive) human breast cancer cells was induced by cultivating the cells in estrogen-free medium and in the presence of doxycycline (1 μg/mL). The level of ER in the ER-positive cells and in wild-type MDA-MB-231 cells was measured using Western blotting and referencing to known concentrations of human recombinant ERα.

Mice and tumors

All experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the Weizmann Institute of Science.

Female CB-17 severe combined immunodeficient (SCID) mice (Harlan Biotech Israel Ltd.), 6 to 7 weeks old, were ovariectomized. About a week later, wild-type human MDA-MB-231 breast cancer cells and stably ER-transfected MDA-MB-231 cells were inoculated (2.5 × 106 cells in 0.1 mL PBS) 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) in 3% sucrose. The size of the tumors was measured by caliper and the volume of the tumors, V, was calculated by the hemiellipsoid equation: V = (length/2 × width/2 × height/2) × 4π/3.

In vivo MRI experiments were carried out 2 to 4 weeks after cell implantation. During the MRI scanning, mice were anesthetized with Isoflurane (Medeva Pharmaceuticals PA, Inc.; 3% for induction, 1%–2% for maintenance) mixed with compressed air (1 L/min) and delivered through a nasal mask. Once anesthetized, the animals were placed in a head-holder to assure reproducible positioning inside the magnet. Respiration rate was monitored and kept throughout the scanning period around 60 to 80 breaths per minute.
Histology
Tumors were fixed in 4% formaldehyde, sectioned parallel to the plane of the MR images (4 μm histologic slice) and stained with hematoxylin and eosin (H&E), as well as immunostained for nuclear ERα, using rabbit monoclonal anti-ER antibody (ER-SP1; Ventana Medical System) and an automated slide staining BenchMark XT system operated according to the manufacturer’s instructions (Ventana Medical System). The overall percentage of ER-stained tumor cell nuclei was estimated by an experienced breast pathologist (A. Yosepovich). In addition, the intensity, I, of staining was determined semiquantitatively as absent (0), weak (1+), moderate (2+), or strong (3+), and the percentage of cells stained at each intensity, I, was estimated. Overall intensity was evaluated using a specific intensity index defined as: \( \sum \text{(intensity} \times \text{fraction of cells stained with intensity)} \).

Biodistribution
Mice were injected with a dose of 0.1 mmol/kg of EPTA-Gd (n = 6), TPTA-Gd (n = 6), or PTA-Gd (n = 5) through the tail vein. About 50 minutes postinjection, mice were euthanized, and brain, uterus, kidney, liver, and muscle tissues were dissected, weighted, homogenized, and lyophilized. The lyophilized samples were treated with concentrated nitric acid overnight, heated to 90°C for 1 hour, and then diluted with deionized water to a final 2% nitric acid. Gd(III) content was determined in the final diluted samples by inductively coupled plasma-mass spectrometry (ELAN 9000 ICP-MS; PerkinElmer). Absolute Gd(III) contents were determined using calibration serial dilutions of Gd standard solution (207136, Gadolinium atomic absorption standard solution, Fluka; Sigma-Aldrich).

MRI
All in vivo MR images were acquired on a 9.4T Biospec AVANCE II spectrometer (Bruker) equipped with a 1H radio-frequency quadrature volume resonator with 72-mm inner diameter. Anatomic information was obtained using multislice T2-weighted, rapid acquisition with relaxation enhancement (factor 8) with echo time/repetition time (TE/TR), 42/3,000 ms; 2 averages; 256 × 128 matrix, reconstructed to a 256 × 256 matrix; field of view, 4 × 4 cm², and slice thickness of 1.2 mm. Subsequently, dynamic contrast enhanced (DCE) MRI experiments were carried out for each contrast agent using a T1-weighted, 3-dimensional gradient-echo (3D-GE) pulse sequence, with TE/TR of 2.5/15 ms; flip angle of 40 degrees. Interleaved axial and coronal images of this sequence were sequentially scanned after a single bolus injection using a spatial resolution in the axial direction of 0.156 × 0.156 mm² and slice thickness of 1.2 mm and in the coronal direction of 0.234 × 0.156 mm² and slice thickness of 1 mm. In the experiments with EPTA-Gd and TPTA-Gd, 4 averages were acquired in each scan (1.5 minutes) and with PTA-Gd, 2 averages were acquired (0.75 minutes). The images recorded in the axial direction monitored signal intensity in the tumors and those recorded in the coronal direction monitored signal intensity in the descending aorta, kidney, and muscle. The jugular veins were scanned separately at a spatial resolution 0.117 × 0.117 × 0.8 – 0.94 mm³ by a T1-weighted 3D-GE sequence (as described above) and a 2-dimensional spin-echo sequence with a constant TE of 8 ms and varying TRs.

Each contrast agent was injected as a bolus into the tail vein of the mice. The dose of EPTA-Gd was 0.1 mmol/kg for blood pharmacokinetic studies (n = 3) and 0.03 mmol/kg (n = 4) or 0.075 mmol/kg (n = 5) for tumor studies. The doses of TPTA-Gd (n = 4) and PTA-Gd (n = 4) were 0.075 and 0.15 mmol/kg, respectively, for both blood pharmacokinetic and tumor studies.

Studies of EPTA-Gd competition with antiestrogens were conducted using tamoxifen citrate salt—TAM (T9262, Sigma-Aldrich) or 4-hydroxytamoxifen—OHT (H7904, Sigma-Aldrich). TAM dissolved in sterile sunflower oil (4 mg/mL) and OHT dissolved in sterile sunflower oil containing 20% ethanol were stirred overnight at 37°C to form homogeneous emulsion. The drug in the oil emulsion was administered by intraperitoneal injection at a final dose of 0.07 mmol/kg TAM (n = 3) or 0.1 mmol/kg OHT (n = 1), 1 hour before the administration of 0.075 mmol/kg of EPTA-Gd. The MRI protocol was the same as described above for the direct, noncompetitive, contrast-enhanced experiments.

Data analysis and statistics
Changes in signal intensity were calculated per pixel yielding enhancement datasets defined as [\( I(t) - I(0) \)/I(0)], where I(0) and I(T) are the signal intensities pre- and postcontrast, respectively. Enhancement maps at pixel resolution were calculated in regions of interest (ROI) in all slices including tumors’ tissue. ROIs were delineated on the anatomic T2-weighted images and transferred to the corresponding DCE images. \( I(0) \) per pixel was calculated as a mean intensity of the 4 precontrast images. Transformation of enhancement units to concentration units was conducted as described in the Supplementary Section. EPTA-Gd and TPTA-Gd concentrations were determined in a whole blood volume, and PTA-Gd concentration was determined in the blood plasma volume, using hemocrit of 0.4 (36).

The time evolution of the concentration in the plasma or blood was fitted to a biexponential decay curve: \( C(t) = D(a_1e^{-m_1t} + a_2e^{-m_2t}) \) where \( a_1 \) and \( a_2 \) are amplitudes and \( m_1 \) and \( m_2 \) are decay rate constants.

Enhancement data sets of ROI in a central slice containing ER-positive and ER-negative tumors recorded during the first 60 minutes were further processed by applying a principal component analysis (PCA) algorithm (see Supplementary Section; ref. 37). Signal enhancement at n time points, \( v^1 \) to \( v^n \), for each pixel \( i \) within the ROI of the ER-positive and ER-negative tumors, were associated with a state vector: \( u_i = (v^1_i, v^2_i, v^3_i, v^n_i)^T \) \( n \), time points; \( T \), transpose). The set of all state vectors in the tumor ROI was defined as \( \Gamma = (u_i) \), \( 1 \leq i \leq N \) where \( N \) is the number of pixels in the ROI. The first-order covariance matrix of \( \Gamma \), COV, was calculated according to:

\[
COV = \frac{1}{N} \sum_{n=1}^{N} (u - \bar{u})(u - \bar{u})^T \text{ where } \bar{u} = \frac{1}{N} \sum_{n=1}^{N} u
\]
A linear PCA transformation by solving $\lambda E = COVE$ was then applied yielding the eigenvectors $E = \{e_1, e_2, \ldots, e_n\}$ and eigen-values $\lambda = \{\lambda_1, \lambda_2, \ldots, \lambda_n\}$. For each pixel, $i$, the state vector $u_i^T$ was then projected on the eigenvectors $E$ so that $u_i^T = p_c;E^T$ where $p_c$ defines the scalar projection coefficients of the eigenvectors $\{pc_1^T, pc_2^T, pc_3^T, \ldots, pc_n^T\}$. The projection coefficient map of each eigenvector $e_i$ was calculated from the scalar values $pc_i^T$ for all pixels within the tumor ROI. Overall, PCA of 8 sets of enhancement maps of each tumor ROI, calculated from the precontrast and 8 consecutive postcontrast intensity scaled data sets, yielded 8 eigenvectors, sorted according to their high to low eigenvalues, as well as 8 corresponding projection coefficient maps for each tumor.

Two-tailed paired $t$ test was applied for comparing average enhancement and average PCA projection coefficient values of ER-positive tumors versus ER-negative tumors, with significant level at $P < 0.05$.

**Results**

**Binding to ER and $T_1$ relaxivity in solution of the ER-targeted probes**

The binding affinity of the 2 ER-targeted probes to the ER and their ability to serve as contrast agents were investigated in solution. The binding inhibition constant to ER, $K_i$ (Table 1; Supplementary Fig. S5) was lower in TPTA-Gd (0.13 $\pm$ 0.006 $\mu$mol/L) than in EPTA-Gd (0.97 $\pm$ 0.07 $\mu$mol/L) reflecting a higher affinity of the former probe. However, both $K_i$ values suggest that binding to ER in cells and tumors in vivo can be achieved using micromolar concentrations of the ER-targeted probes. The water proton T1 relaxivity in solution of the 2 targeted probes (Table 1) showed that EPTA-Gd exhibited a higher T1 relaxivity (6.8 $\pm$ 0.7 (L/mmol)/s) than TPTA-Gd (4.7 $\pm$ 0.1 (L/mmol)/s), PTA-Gd which served as a control contrast agent for assessing the microvascular perfusion capacity of the tumors exhibited the lowest relaxivity (3.0 $\pm$ 0.1 (L/mmol)/s) in accordance with the small size of this agent.

**Detecting ER in breast cancer in vivo with the ER-targeted probes**

**Characterization of tumors**

ER-transfected MDA-MB-231 cells cultivated in estrogen-free medium and induced to produce ER by doxycycline (1 $\mu$g/mL) exhibited high ER levels of 11,000 to 14,000 fmol/mg protein (2 independent experiments). In contrast, the level of ER in the wild-type MDA-MB-231 cells was null (Supplementary Fig. S6). The ability of the targeted probes to detect ER was evaluated by monitoring the enhancement they induced in tumors of ER-transfected (ER-positive) and wild-type (ER-negative) MDA-MB-231 cells, implanted in the same mouse. Solid, palpable ER-positive and ER-negative tumors developed within a week after the inoculation of cells into the mammary fat pad of the SCID mice. Both types of tumors continued to grow exponentially (Fig. 2A) at a similar rate ($P > 0.9$, paired $t$ test, $n = 10$) reaching after 22 days a size of 200 to 250 mm$^3$. H&E-stained sections of these tumors exhibited the presence of viable cells as well as nuclear dust of dead cells in both ER-positive and ER-negative tumors (Fig. 2B). ER immunostaining showed a strong ER nuclear staining in viable areas of ER-positive tumors (Fig. 2C) and null or very low staining of ER in the ER-negative tumors (Fig. 2D). Quantitative analysis indicated that the percentage of ER-stained tumor cell nuclei
was 71.0% ± 0.05% in the ER-positive tumors and 1.0% ± 0.03% in the ER-negative tumors. This difference was also reflected in the specific intensity index of ER-positive tumors of 2.12 ± 0.01 as compared with 0.02 ± 0.08 in the ER-negative tumors (n = 11 in each group). These results are in accordance with the ER levels determined in the same cells cultivated in estrogen-free culture medium (Supplementary Fig. S6).

**Clearance from blood and kidneys and biodistribution in various tissues**

The pharmacokinetics of the 2 targeted probes and the nontargeted PTA-Gd in the blood and their clearance from the kidney was followed by MRI and converted to concentration units as described in Materials and Methods. The blood concentration time courses of all 3 agents indicated an initial rapid decay followed by a slow decay (Fig. 3A–C). The fast decay constant, $m_1$, was highest for PTA-Gd and decreased slightly for EPTA-Gd and more substantially for TPTA-Gd ($m_{PTA-Gd} = 0.54 ± 0.18 \text{ min}^{-1}, n = 4; m_{EPTA-Gd} = 0.37 ± 0.29 \text{ min}^{-1}, n = 3; m_{TPTA-Gd} = 0.17 ± 0.06 \text{ min}^{-1}, n = 4$). The slow decay constant, $m_2$, was similar for PTA-Gd and EPTA-Gd but decreased by 10-fold for TPTA-Gd ($m_{PTA-Gd} = 0.04 ± 0.02 \text{ min}^{-1}, n = 4; m_{EPTA-Gd} = 0.03 ± 0.02 \text{ min}^{-1}, n = 3; m_{TPTA-Gd} = 0.003 ± 0.002 \text{ min}^{-1}, n = 4$). The marked slow down in the second clearance phase of TPTA-Gd is in accordance with the high accumulation of this agent in muscle tissue (see below) which comprises a large fraction of the body mass, thereby slowing down the overall clearance. These results indicated

Figure 3. Pharmacokinetics and biodistribution of EPTA-Gd, TPTA-Gd, and PTA-Gd in SCID mice. A–C, kinetics of EPTA-Gd and TPTA-Gd concentration in blood ($C_b$) and of PTA-Gd concentration in plasma ($C_p$). The agents were administrated as bolus injection into the tail vein of CB-17 SCID mice at the following doses: EPTA-Gd, 0.1 mmol/kg; TPTA-Gd, 0.075 mmol/kg; PTA-Gd, 0.15 mmol/kg. T1-weighted, gradient-echo MR images of a major blood vessel were recorded sequentially. EPTA-Gd kinetics was conducted in a separate set of experiments from the contrast-enhanced studies of the tumors, monitoring the jugular vein. TPTA-Gd and PTA-Gd kinetics were monitored in descending aorta analyzing coronal images scanned together with the scanning of the tumors. Signal enhancement was converted to blood concentration units and then fitted to a biexponential decay as described in the Materials and Methods and in Supplementary Data. The decay rate constants in the blood and plasma are given in the text. D–F, the time courses of enhancement in the kidney cortex. G, biodistribution of EPTA-Gd, TPTA-Gd, and PTA-Gd in CB-17 SCID mice. The Gd(III) content was examined by ICP-MS at 50 minutes postinjection of a dose of 0.1 mmol/kg of each contrast agent into the tail vein. Data presented as mean ± SD of n = 6 for EPTA-Gd and TPTA-Gd and n = 3 for PTA-Gd.
fast blood clearance of PTA-Gd, typical to external contrast agents, fast blood clearance of EPTA-Gd and a very slow, delayed clearance of TPTA-Gd.

The time courses of enhancement in the kidneys also revealed marked differences between the agents (Fig. 3D–F): PTA-Gd and EPTA-Gd clearance was relatively fast with a half-life of approximately 20 and 40 minutes, respectively, whereas the half-life of TPTA-Gd was longer than the measurement time of 140 minutes. Clearly, the time courses of TPTA-Gd in both the blood and kidney indicated that this agent is sustained in the body for a much longer period than the other 2 agents.

The above results were further supported by measuring the Gd(III) content in various tissues using inductively coupled plasma-mass spectrometry (ICP-MS). The contrast agent PTA-Gd cleared out from most tissues at 50 minutes and was predominantly found in the kidney (Fig. 3G), indicating a pharmacokinetics similar to the common Gd-based extracellular contrast agents (38). EPTA-Gd was predominantly found in the liver in accordance with the liver function as the major chemical detoxifier in the body, with about 10-fold decreased levels in the kidney and uterus and very low levels in the muscle and brain (Fig. 3G). TPTA-Gd also accumulated predominantly in the liver; however, unlike EPTA-Gd, substantial accumulation occurred also in the kidney, uterus, and muscle with low accumulation only in the brain (Fig. 3G).

Interestingly, throughout the experiments that required in vivo injection of the new contrast agents, no toxic effects on the mice were observed. Although no rigorous toxicity evaluation were conducted, it appeared that the ER-targeted probes and the nontargeted probe did not cause abnormal changes in the behavior and health of the mice at doses ranging between 0.03 and 0.15 mmol/kg.

**In vivo MRI of ER-positive and ER-negative tumors**

The exact size and the anatomic features of the tumors were examined before the administration of the targeted probes by recording T2-weighted images (Fig. 4A). The MRI results confirmed the caliper measurements (Fig. 2A) showing no significant size difference between the ER-positive and ER-negative tumors ($P = 0.91$, $n = 17$; see also Supplementary Table S1).

The time courses of contrast enhancement induced by the targeted probes and PTA-Gd showed distinct patterns in the ER-positive and ER-negative tumors. EPTA-Gd induced a consistently and significantly higher enhancement in the ER-positive tumors than in ER-negative tumors (Fig. 4; Table 2). In contrast, comparable enhancement ($P > 0.2$) was found in both types of tumors after injecting TPTA-Gd or PTA-Gd (Fig. 4; Table 2), suggesting an absence of specific interaction or binding of these agents with ER. The enhancement induced by EPTA-Gd in the ER-positive tumors was also significantly higher than that in muscle tissue (Fig. 4C). For example, at 40 to 45 minutes post–TPTA-Gd injection, the mean enhancement in the muscle (13.7 ± 3.3) was significantly lower ($P < 0.005$, $n = 9$) than in the ER-positive tumors (39.7 ± 17.7).

Overall, the enhancements in muscle and ER-negative tumors were not significantly different suggested using muscle tissue as a reference for a nonspecific interaction of EPTA-Gd when testing tumors for their ER level. The muscle enhancement induced by PTA-Gd was lower than the tumors’ enhancement and similar to the enhancement induced by the common Gd-based extracellular contrast agents (38, 39), in accordance with the perfusion parameters of muscle tissue. Unexpectedly, TPTA-Gd induced a higher enhancement in the muscle than in the tumors, suggesting a specific binding of this agent to a muscle component (Fig. 4C). For example, at 40 to 45 minutes post–TPTA-Gd injection, the mean enhancement in the muscle ($28.5 ± 6.4$) was significantly higher ($P < 0.01$, $n = 4$) than in the ER-positive tumors ($12.2 ± 1.3$).

Further quantitative evaluation of the enhancement curves using model-free PCA provided a strong evidence for the augmented accumulation and interaction of EPTA-Gd with ER in vivo. The PCA data sets produced 8 eigenvectors arranged according to their eigenvalues (Fig. 5A; Supplementary Fig. S7), with the first eigenvector with the highest eigenvalue depicting the variance because of the enhancement caused by the various agents (Fig. 5B).

The second eigenvector, with approximately 2 orders of magnitude lower eigenvalue, was less reproducible than the first eigenvector for the same contrast agent and appeared to depict variances because of a decline in enhancement mixed with noise (Fig. 5C). The remaining 6 eigenvectors appeared to depict variances because of noise fluctuations for all contrast agents (Supplementary Fig. S7). The projection coefficient parametric maps of the EPTA-Gd–induced first eigenvector (Fig. 5E) were significantly higher in the ER-positive tumors than in the ER-negative ones ($P = 0.007$; Table 2), enabling detection of ER expression. The projection coefficients of the EPTA-Gd–induced second eigenvector were negative, close to null and similar in both ER-positive and ER-negative tumors depicting slow contrast clearance (Table 2). The remaining projection coefficients were also close to null and similar in both tumors as expected for noise fluctuations.

As was found by the enhancement analysis, the projection coefficient values of the first and second eigenvector because of TPTA-Gd and PTA-Gd injection (Fig. 5E; Table 2) showed no significant differences between ER-positive and ER-negative tumors, neither did all other projection coefficients depicting the noise variance. Thus, a full congruence was obtained between the results obtained by quantifying the enhancement and by PCA, showing the ability of EPTA-Gd to identify ER-positive tumors through their augmented enhancement.

Further confirmation of the distinct ability of EPTA-Gd to bind selectively ER in vivo was obtained by blocking this receptor in the ER-positive tumors with tamoxifen or 4-hydroxymoxifen. The intraperitoneal injection of these antiestrogens, an hour before conducting a DCE experiment with EPTA-Gd, showed that both ER-positive and ER-negative tumors exhibit similar enhancement time curves (Fig. 4D). Statistical analysis ($n = 4$) indicated no significant differences in the enhancement between ER-positive and ER-negative tumors at 20 to 25 minutes (mean ± SD of ER-positive and ER-negative tumors were $38.4 ± 16.6$ and $29.1 ± 12.2$, respectively; $P = 0.54$), as well as at 40 to 45 minutes (mean ± SD of ER-positive and ER-negative tumors were $28.7 ± 5.1$ and $29.4 ± 9.5$, respectively; $P = 0.95$). Furthermore, the enhancement in the ER-positive tumors were not signifi
tumors blocked with the antiestrogens was not significantly different from the muscle enhancement at 20 to 25 minutes (mean ± SD of muscle was 31.2 ± 7.0; \( P = 0.36 \)), as well as at 40 to 45 minutes (mean ± SD of muscle was 25.6 ± 6.5; \( P = 0.43 \)).

**Discussion**

The assessment of ER levels in breast cancer is currently conducted by means of *ex vivo* methods that examine a small sample fraction of the lesion. Developing novel molecular imaging methodologies for detecting and mapping this receptor *in vivo*, throughout the entire tumor volume, can facilitate the management of patients with breast cancer at the various stages of diagnosis and treatment. In this work, we have examined and showed the potential of 2 newly designed ER-targeted probes containing a paramagnetic Gd chelate to image the ER *in vivo*, in a human breast cancer mouse model. We also compared the performance of these 2 probes with that...
of a nontargeted contrast agent composed of the same Gd chelate as the targeted probes.

Studies in solution clearly showed that the 17β-estradiol or tamoxifen moiety of the new contrast agents enabled a high binding affinity with the purified ER in solution, in the micromolar range, with the affinity of TPTA-Gd approximately 3-fold higher than that of EPTA-Gd. The ability of these 2 probes to augment T1 relaxation of water was reversed; EPTA-Gd relaxivity is approximately 1.5-fold higher than that of TPTA-Gd and 2.5-fold higher than PTA-Gd relaxivity. The differences in the paramagnetic-induced T1 relaxivities could stem from the variations in the molecular weight and structure, affecting the tumbling rates of these molecules, as well as the strength of the dipolar interaction of the first and second sphere water molecules with the paramagnetic Gd (40).

To characterize the in vivo efficiency of these agents to interact with ER, we monitored the MRI enhancement time courses caused by these agents in ER-positive tumors, expressing high ER level in the tumors due to tetracycline-induced upregulation of this receptor, and in ER-negative breast tumors of the same origin, with close to null ER levels, both implanted in the same mouse. The level determined in the ER-positive cultured cells was approximately 10,000 fmol/mg protein. Assuming that proteins contribute to the cells’ weight approximately 10% to 20%, a rough estimation of ER concentration in the ER-positive cells, of 1 to 2 approximately 10% to 20%, a rough estimation of ER concentration in the cultured cells was approximately 10,000 fmol/mg protein.

Assuming that the level determined in the ER-positive cultured cells was approximately 10,000 fmol/mg protein, we calculated the concentration of ER in the ER-positive tumors, of 1 to 2 μmol/L, can be obtained. This concentration represents an upper bound on ER concentration in ER-positive tumors, as the tumors are not solely composed of cells and include an extracellular compartment void of ER. Furthermore, ER concentration in some regions of the ER-positive tumors could be appreciably lower than this upper limit as suggested from the inhomogeneity of ER distribution revealed in the immunostained slides and in the enhancement maps.

Further characterization of the biodistribution of these agents in various organs was obtained by MRI monitoring of the blood, kidney, and muscle, as well as by means of ICP-MS. The enhancement patterns of PTA-Gd in the blood, kidney, and muscle were expected to be similar to those of the common extracellular Gd-based contrast agents such as GdDTPA (38). Indeed, PTA-Gd behaved like an extracellular contrast agent and showed rapid clearance from all tissues and blood into the kidneys and urine. In addition, the time course and spatial distribution of PTA-Gd in both types of MDA-MB-231 tumors were not significantly different and were comparable with those reported for GdDTPA, suggesting disparity in the influx and outflux transcapillary transfer rates (41).

Both EPTA-Gd and TPTA-Gd appeared to enter cells and accumulate intracellularly in the tumors, maintaining a sustained level during the entire 150 minutes of the measurements. The ICP-MS results clearly showed that the liver served as the main sink for the accumulation of these 2 ER-targeted probes, in accordance with its function as the major chemical detoxifier in the body. However, the clearance of these 2 agents from the blood and the kidneys and their accumulation in various organs were highly diverse: EPTA-Gd cleared out from the blood much faster than TPTA-Gd and did not accumulate to a large extent in muscle tissues, whereas TPTA-Gd cleared very slowly from the blood and accumulated to high levels in muscle tissues. The augmented muscle accumulation of the nonsteroidal tamoxifen-derived contrast agent appears to be due to strong interaction and possibly binding with cellular muscle components. A supporting evidence for such an explanation is the binding of tamoxifen to calmodulin (42), which regulates in skeletal muscle, the enzymatic activity of myosin light chain kinase (43).

The most relevant difference between EPTA-Gd and TPTA-Gd about their ability to detect ER was the finding that PTA-Gd−induced enhancement was significantly higher in ER-positive tumors than in ER-negative tumors, whereas TPTA-Gd−induced enhancement was very low and not significantly different in the 2 tumor types. It is well known that binding of a Gd-based contrast agent to macromolecules increases the rotational correlation time and leads to increased relaxivity of

| Table 2. EPTA-Gd, TPTA-Gd, and PTA-Gd–induced enhancement (%) over whole tumor volume at 2 time points postcontrast and projection coefficient of the first and second eigenvectors of ER-positive and ER-negative tumors |
|----------------------|----------------------|----------------------|----------------------|----------------------|
|                     | **Enhancement, %**    |                     | **Projection coefficient** |
|                     | **At 20–25 min**      | **At 40–45 min**    | **First eigenvector**     | **Second eigenvector** |
|                     | Positive | Negative | Positive | Negative | Positive | Negative | Positive | Negative |
| EPTA-Gd, n = 9      | 46.1 ± 21.4 | 28.3 ± 2.6 | 39.7 ± 17.7 | 26.7 ± 6.1 | 1.0 ± 0.6 | 0.35 ± 0.3 | −0.05 ± 0.08 | −0.04 ± 0.08 |
| 10.9 ± 4.5          | 13 ± 17.6 | 12.2 ± 1.3 | 19.4 ± 10.4 | 0.33 ± 0.15 | 0.29 ± 0.3 | −0.16 ± 0.07 | −0.04 ± 0.12 |
| 3.3 ± 1.0           | 0.8 ± 0.9 | 0.34 | 0.8 ± 0.5 | 0.77 ± 0.3 | 0.07 ± 0.2 | −0.1 ± 0.3 |

NOTE: Values presented as mean ± SD; n indicates number of experiments.

*Significant difference based on two-tailed paired t test.
the exchanging bulk water (44). Thus, the distinct high enhancement in ER-positive tumors induced by EPTA-Gd served to indicate interaction and binding of this probe to ER in vivo. An additional confirmation that indeed the higher enhancement induced by EPTA-Gd in ER-positive tumors is due to interaction with ER was obtained by in vivo competition experiments with TAM and OHT. TAM and OHT exhibit higher affinity to ER than EPTA-Gd and were administered at a relatively high dose to ensure blockage of ER. Consequently, the antiestrogen blockage of ER caused the EPTA-Gd–induced enhancement in the ER-positive tumors to reflect a low, unspecific enhancement, as was found for the ER-negative tumors.

The in vivo differences between EPTA-Gd and TPTA-Gd were unexpected in view of the solution ER-binding affinity and relaxivities of these 2 probes described above. However, the fact was that a large fraction of TPTA-Gd accumulated in muscle tissues could reduce the effective dose that reached the tumors below the micromolar range necessary for the augmentation of enhancement. Thus, it appears that in contrast to EPTA-Gd, fast uptake and accumulation of TPTA-Gd in muscle tissues limited its ability to serve as a targeted ER probe in vivo.

Differences in enhancement maps and projection coefficient maps of the ER-positive and ER-negative tumors could result from differences in ER level and cellular viability, as well as from variations in the tumors’ perfusion parameters such as microvascular flow, permeability multiplied by surface area, and extent of interstitial fluid pressure. The application of the nontargeted PTA-Gd indicated a high congruence in these parameters in both tumor types, suggesting that the differences in the DCE of EPTA-Gd are not due to the perfusion parameters but most likely stem from the intracellular difference in the ER level.

It is important to note that although the enhancement patterns of both PTA-Gd and TPTA-Gd were similar in both the ER-positive and ER-negative tumors, the patterns of enhancement induced by each agent was different, as expected from the difference in their ability to enter cells. PTA-Gd underwent fast clearance in both tumor types, like most extracellular contrast agents, whereas the low enhancement induced by TPTA-Gd remained stable for the duration of the experiment possibly due to the slow clearance of this agent.

In conclusion, the 17β-estradiol–derived contrast agent EPTA-Gd was found to specifically and significantly enhance the MR signal in ER-positive tumors as compared with ER-negative tumors or muscle tissue, showing a feasibility to be applied as a targeted probe for molecular ER imaging in vivo. Blocking the ER by antiestrogens reduced the enhancement in the ER-positive tumors to the nonspecific enhancement level found in ER-negative tumors and muscle.
confirmed the specific interaction of EPTA-Gd with ER in the direct experiments without competition. Thus, detecting in tumors, EPTA-Gd–induced enhancement, at significantly higher level than muscle enhancement, may serve for detecting, localizing, and monitoring ER noninvasively. In addition, further characterization of the cellular interactions of tamoxifen-derived probes may shed light on the specific interactions of this antihormone with cellular components other than ER.

Disclosure of Potential Conflicts of Interest

All authors have no potential conflicts of interest to disclose with regard to this work.

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In Vivo Magnetic Resonance Imaging of the Estrogen Receptor in an Orthotopic Model of Human Breast Cancer

Adi Pais, Chidambaram Gunanathan, Raanan Margalit, et al.

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