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Rational Design, Synthesis, and Biological Evaluation of Progesterone-Modified MRI Contrast Agents

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SUMMARY

A series of contrast agents for magnetic resonance imaging (MRI) aimed at noninvasively determining the hormone receptor status of cancer in vitro was developed. These MRI contrast agents were prepared by conjugating progesterone to clinically used Gd(III) chelates. These agents exhibited higher progesterone receptor binding affinities in the nanomolar range and intracellular accumulation. High log*P* values of the modified compounds suggested that the lipophilicity of the steroid conjugates may have contributed to membrane permeability. Synchrotron radiation X-ray fluorescence microscopy and magnetic resonance images revealed that the synthesized conjugates showed the greatest cellular accumulation and significant increase in relaxivity in vitro compared to the previously developed steroid-modified agent. Transcriptional assays using the progesterone response element linked to luciferase indicated that the contrast agents entered the cell, interacted with the biological target, and drove specific progesterone-mediated transcription.

INTRODUCTION

Magnetic resonance imaging (MRI) has become an important tool in the clinical diagnosis of diseases. Images are acquired by employing radio frequency pulses to excite nuclear spins of a specimen and imposing one or more orthogonal magnetic field gradients [1, 2]. The observed signal is from the protons of water molecules, where signal intensity in a given volume element is a function of the water concentration and relaxation times (T_1 and T_2) [3]. Optical microscopy of opaque specimens is limited by light scattering, whereas MRI can image in three dimensions with high spatial and temporal resolution [4, 5]. Intrinsic MR contrast can be enhanced by using agents that modulate the spin-lattice relaxation rates of water protons [6]. Paramagnetic ions such as Gd(III) are routinely used to decrease the local T_1 relaxation of nearby water protons, and when chelated are nontoxic contrast agents [6].

A barrier to the further development of MRI as a diagnostic tool is the lack of targeted contrast agents. New classes of bioactivatable agents have been prepared to reduce background signal and possess two distinct states (on and off) as a result of the absence or presence of enzymes or secondary messengers [7–10]. Numerous examples have been reported where the agents are loaded into cells or conjugated to molecular targeting platforms such as monoclonal antibodies [11]. However, these approaches are limited by the ability of the agent to be linked to an antibody without perturbing the recognition properties or limited by the amount of uptake into cells. Therefore, targeted strategies for new generations of chelates and ligands are required to improve cellular permeability and specificity of MRI agents.

The progesterone receptor (PR) is a member of the nuclear receptor superfamily that functions as a ligand-activated transcription factor. Mammary epithelial cells express the PR and estrogen receptor (ER) [12]. The PR is present in two distinct isoforms both derived from the same gene, PRA and PRB. Each subtype is critical to mammary gland lobuloalveolar development and epithelial differentiation [13]. The receptor consists of several regions that serve as functional units such as the DNA binding domain, the ligand binding domain, and transcriptional activation domains. The expression of these receptors is a critical parameter typically examined using immunohistochemistry in biopsies of human breast cancers [14, 15]. The presence of both receptors correlates with the survival rate of breast cancer patients [16]. The PR is an estrogen-regulated gene that becomes activated and expressed in the presence of estradiol and ER. It is not surprising that treatment with tamoxifen reduces PR and correlates with tamoxifen resistance, although the mechanism of resistance is still debated [17]. Tumors that are ER+/PR− are considered more aggressive than PR+ tumors and correlate with a lower survival rate [18, 19]. The current clinical methods to determine PR and ER levels require tissue biopsy or radioisotope injection followed by ionizing radiation. Therefore, noninvasively determining whether or not a mammary cancer expresses PR may be crucial to deciding the most effective chemotherapeutic agent for the patient.

As a result of the hydrophilic nature of Gd(III) chelates, MR contrast agents do not traverse cell membranes and are restricted to the extracellular domains [10, 20]. These chelates are thermodynamically stable ($K_d = 10^{-21} \sim 10^{-25}$) and kinetically inert [6]. Steroids readily diffuse across the phospholipid bilayer due to their hydrophobic properties and small size [21]. Further, the availability of receptor-specific hormones could determine whether the steroid is retained within a specific cell type allowing the cell, and not the contrast agent, to determine molecular targeting. The steroid progesterone is an endogenous molecule with limited toxic activity and well-established pharmacokinetic profiling [22]. In addition, steroids are typically retained in the nucleus of cells once bound to their receptor, where they interact with the DNA to drive gene transcription. Contrast agents that bind to large macromolecules such as enzymes or proteins undergo a dramatic increase in the relaxation rate of nearby water protons [1, 2]. Binding to a macromolecule increases concentration and retention of the Gd(III) complex at the receptor binding site and affords an increase in rotational correlation time (τ_r) of the agent [6]. Therefore, progesterone-Gd(III) chelate conjugates are an attractive choice for use as MRI contrast agents that target hormone receptor-positive cells [23].

Steroid-conjugated magnetic resonance contrast agents are a rational design for generating targeted enhancement due to intracellular accumulation. As breast tumors become more aggressive, they typically lose hormone receptors and become less responsive to hormone-based breast cancer therapies. Few methods are available for imaging breast tumors in vivo, and none facilitate the molecular or therapeutic profiling of tumors. Previously, RU-486-modified MR contrast agents were proved to be membrane permeable, and successfully interact with progesterone receptors in cells and provide limited signal enhancement [23]. Therefore, the purpose of this investigation was to develop Gd(III)-conjugated steroid contrast agents that accumulate intracellularly, where they might interact with nuclear progesterone receptors and thereby increase magnetic resonance. By using a progesterone receptor targeted contrast agent, it may be possible to visualize the morphology of organs and diseased tissue and to determine the biochemical characteristics of cells. Finally, a synthetic methodology has been designed to prepare new MRI contrast agents that can be targeted to other hormones for imaging of hormone-dependent cancers [24].

RESULTS

Design and Synthesis of Progesterone Receptor Targeted MR Contrast Agents

A series of progesterone conjugates with Gd(III) contrast agents was synthesized and characterized (Figure 1). Previously, RU-486 was modified with a similar Gd(III) chelate, and it was discovered that the site of attachment was critical to the binding affinity of the complex. The RU-486-modified conjugate had approximately a 100-fold decrease in affinity for the receptor [23]. As a result of those experiments, the labeling strategy was modified for the synthesis of new progesterone agents. The 3-keto group on the hormone is not an ideal modification site because it interacts with a highly conserved region in the receptor protein [25]. Therefore, modification or isomerism at this site may compromise the binding affinity of the modified steroid [26–28]. A number of alternatives to this site are available and position 17 of the D ring was chosen.

A series of Gd(III) complexes was synthesized to examine the effect of charge on lipophilicity and cell permeability of the conjugate (neutral, −1, and −2). Further, to determine the effect distance between the chelate and steroid may have on receptor binding affinity, spacers with varying lengths (zero, three, and six methylene carbons) were inserted between progesterone and the Gd(III) chelate. The neutral series of conjugates with zero- and six-carbon spacers (**1**, **2**) was synthesized from 21-hydroxyprogesterone, as shown in Figures 2A and 2B. The synthesis of **1** began with bromination of the 21-hydroxyl group using carbon tetrabromide and triphenylphosphine. The attachment of **5** with K_2CO_3 (NBu_4OH as a catalyst) afforded the t-butyl-protected ligand (**6**) in high yield (93%). After deprotection of the t-butyl group by trifluoroacetic acid, the ligand was heated with Gd(III) chloride at 60°C to produce compound **1** in 72% yield. The synthesis of **2** started with alkylation of the 21-hydroxyl group by 1,6-dibromohexane. This biphasic alkylation reaction produced bromine-tethered progesterone (**7**) in 74% yield. A coupling reaction with DO3A followed by insertion of the lanthanide was performed by the same methods described in the synthesis of **2**, with 58% yield.

The synthesis of the charged series of conjugates (**3**, **4**) begins with the bromine intermediates (**5**, **7**) from the neutral series (Figure 2C). In the first step, the bromine group was substituted with an azide. After reducing the azide with Lindlar's catalyst, a coupling reaction of the free amine with an isothiocyanate-Gd(III) chelate was attempted. However, when the pendant amine is neutral or in basic form, the product is unstable, producing a number of uncharacterized byproducts. To circumvent this problem, the azide group was reduced and protected with a Boc group in one pot to give **13** and **14**. After deprotection of the Boc group using trifluoroacetic acid, the TFA salt of amines was coupled with the charged Gd(III) chelates (**15** and **16**) to produce **3** and **4**, respectively. Chelates possessing a −1 or −2 charge with no spacer (**17** and **18**) decomposed during preparative high-performance

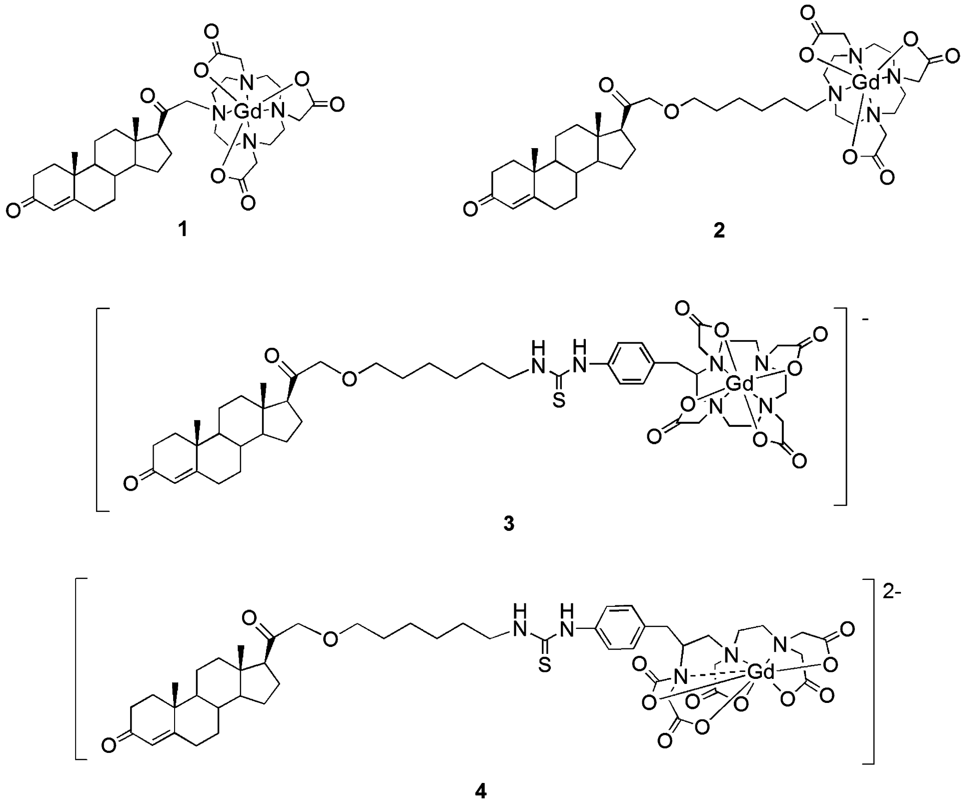


Figure 1. Structures of Progesterone-Conjugated MRI Contrast Agents

liquid chromatography purification, and the structure of these molecules was not determined. The charged Gd(III) chelates (**15** and **16**) were synthesized from commercially available ligands by previously published research [23].

Relaxivity and Octanol-Water Partition Coefficients

The relaxivity and octanol-water partition coefficients of **1**–**4** are presented in Table 1 and show that charged complexes have higher relaxivities. This tendency is due to aggregation caused by the amphiphilic nature of the compounds. The evidence supporting complex aggregation is the high relaxivity of **3** (19.1 $mM^{-1}s^{-1}$) measured in the range of 0.125–2 mM. However, when the solution was diluted approximately 100-fold (concentration range from 0.001 to 0.1 mM), the relaxivity decreased to 5.9 $mM^{-1}s^{-1}$.

The octanol-water partition coefficient (P) of each compound was measured to determine lipophilicity. The P value is often expressed in logarithmic form (log$P$), because the values usually range over many orders of magnitude. The observed log$P$ values of the progesterone conjugates indicate that aggregation is occurring. Compound **3** has approximately the same log$P$ value as **1**; however, the relaxivities of these compounds are very different (19.1 and 3.77 $mM^{-1}s^{-1}$, respectively). The results indicate that the overall lipophilicity of the two molecules is similar and that the charged species seem to be sufficiently amphiphilic to aggregate in solution. In addition, cellular uptake studies show that unlike commercially available Gd(III) contrast agents, such as Prohance, these new steroid conjugates are membrane permeable. Compared to Prohance (log$P$ = -2) [29], all conjugates have greater log$P$ values, implying that lipophilicity of the molecules may contribute to membrane permeability.

Steroid-Gd(III) Conjugates Bind to the Ligand Binding Domain of Progesterone Receptor A

Modification of a steroid with a Gd(III) chelate may interfere with the interaction of the steroid with its receptor. To examine the effect of the modification, competitive binding experiments were performed using increasing doses of the contrast agents and fluorescently labeled progesterone as the competitor. At least five doses for each compound were used to determine the IC_{50} of each compound for progesterone receptor. The results are shown in Table 2. Relative binding of each progesterone contrast agent can be compared to progesterone. Compound **1** had the highest relative binding affinity for the receptor of all the compounds and differed only slightly from compound **3**. Compound **2** had a relative affinity for the receptor that was about 100-fold lower than progesterone alone, but this was still in the high nanomolar range.

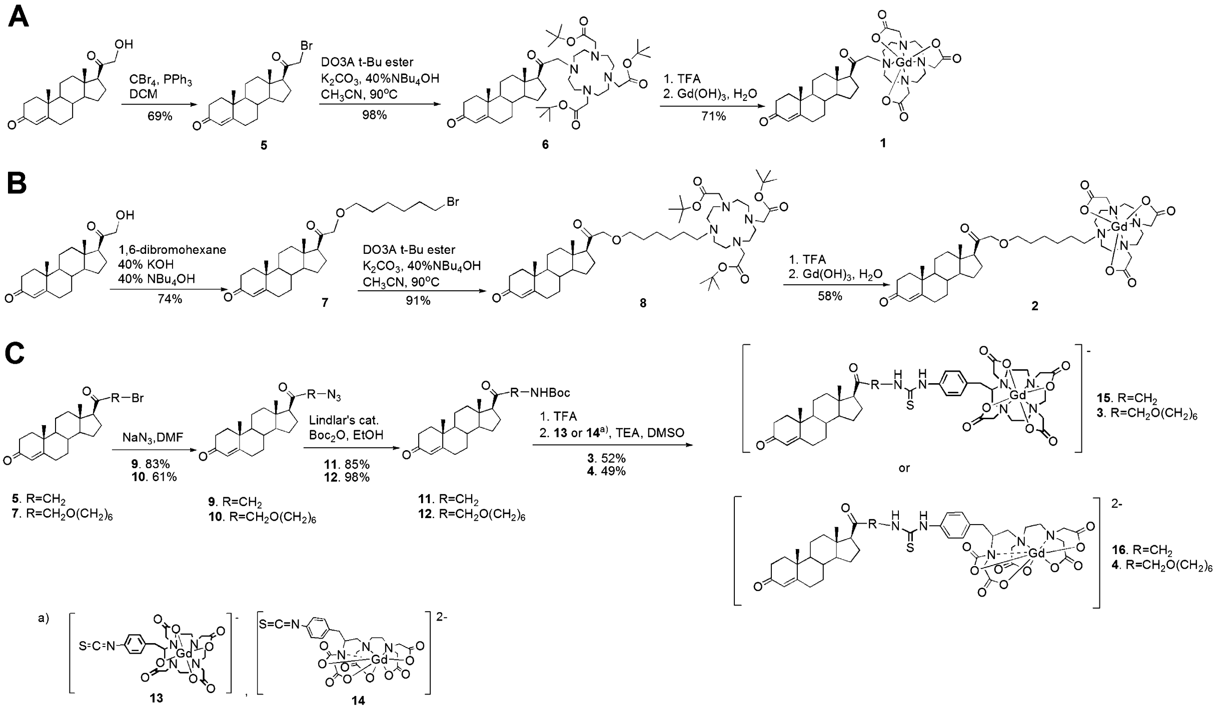


Figure 2. Synthesis of Progesterone-Modified Gd(III) Chelate Conjugates

(A) Synthesis of neutral conjugates containing no spacer between the steroid and contrast agent.

(B) Synthesis of neutral conjugates containing a six-carbon spacer.

(C) Synthesis of charged progesterone conjugates.

Steroid-Based Contrast Agents Demonstrate Accumulated Gd(III) Inside the Cell

To determine the cell permeability and toxicity of the compounds, cellular uptake experiments were performed by incubating the contrast agents with progesterone receptor-positive (T47D) and progesterone receptor-negative (MDA-MB-231) mammary epithelial cells. Dose-response experiments were performed to identify the proper concentration of contrast agents to accomplish cellular accumulation. The cells were incubated with 50, 5, 0.5, or 0.05 μM agents for 24 hr with T47D and MDA-MB-231 cells. The contrast agents were absorbed in a dose-dependent manner and demonstrated maximal accumulation at 50 μM (Figure 3A). Cells were incubated with only **1** or **2** at different time points including 1, 2, 4, and 24 hr to determine the optimal incubation time for accumulation. After incubation, the cells were lysed and subjected to inductively coupled plasma mass spectrometry (ICP-MS) analysis to quantify the number of Gd(III) ions present in each cell based on milligrams of protein (Figure 3B). Compound **2** showed the highest uptake into the PR-expressing T47D cell line, and in cells lacking PR, the MDA-MB-231 cell line. Compounds **3** and **4** had much lower uptake, indicating that the negative charge of the contrast agent reduces cellular permeability.

Table 1. Relaxivity Data and Octanol-Water Partition Coefficients (Log*P*) of 1–4

Compound	Log*P*	Relaxivity ($mM^{-1}s^{-1}$)[a]
Progesterone	3.87[b]	—
1	−0.292	3.77/4.76[c]
2	0.262	4.73[c]
3	−0.377	19.1/5.9[d]
4	−0.959	6.5
Prohance	−2.0	3.7[e]

[a] Data were measured at 60 MHz, 37°C.
[b] Data were taken from Alvarez Nunez and Yalkowsky [29].
[c] Data were measured at 4.7 T, 21°C.
[d] Data were measured at low concentration from 0.001 to 0.1 mM.
[e] Data were taken from Caravan et al. [6].

Table 2. Receptor Binding Affinity Results of 1–3

Compound	IC_{50} (M)
Progesterone	1.6×10^{-9}
1	9.6×10^{-8}
2	4.6×10^{-7}
3	8.6×10^{-7}
RU-486[a]	2.2×10^{-8}
RU-486-Gd[a]	1.9×10^{-6}

[a] Data were taken from Lee et al. [23].

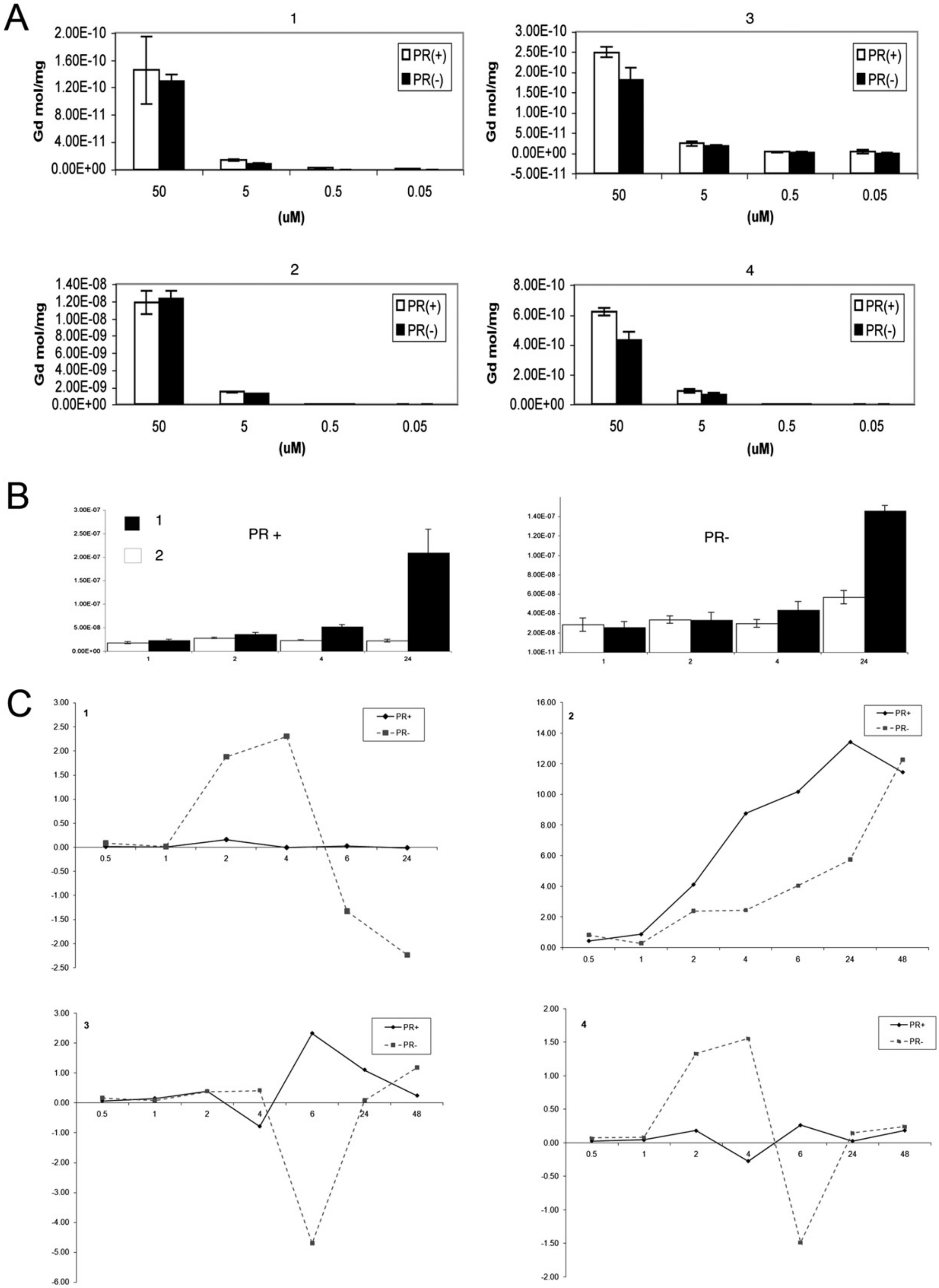


Figure 3. Cellular Uptake Studies of Progesterone-Modified Contrast Agents

(A) Progesterone Gd(III) chelates are dose dependently absorbed into mammalian breast cancer cells that either express progesterone receptor or are receptor negative. Cells were incubated with 50, 5, 0.5, or 0.05 μM contrast agents for 24 hr, washed with PBS, and lysed, and the amount of

Similarly, compound **2** was significantly more absorbed than **1**, demonstrating the importance of the hydrophobic carbon linker. To determine the cellular distribution of **1** and **2**, synchrotron radiation X-ray fluorescence (SR-XRF) analysis was performed. SR-XRF spectroscopy uses high-energy X-rays to produce a map of each element's concentration with submicrometer resolution, whereas conventional XRF analysis provides the elemental composition of materials. A major advantage of SR-XRF over standard fluorescence microscopy is that images are obtained without altering the agent by attachment of an organic fluorophore [22]. Compounds **1** and **2** (50 μM) were incubated with progesterone receptor-positive cells (T47D) and -negative cells (MDA-MB-231) for 24 hr prior to scanning. The samples were raster scanned at coarse resolution (2.0 μm × 2.0 μm step size). The images show accumulation of each compound within cells and confirmed cellular uptake of **2** (see Supplemental Data available with this article online).

Steroid-Based Contrast Agents Efflux More Slowly from Receptor-Expressing Breast Cancer Cells

One aspect of molecular specificity that a steroid-based contrast agent may provide is retention in cells expressing the progesterone receptor. Because progesterone interacts with its receptor and is active in the nucleus as a transcription factor long after initial absorption, experiments were designed to determine whether this would result in a slower leaching of the contrast agents from progesterone receptor-expressing T47D cells as compared to the progesterone receptor-negative MDA-MB-231 cells. Cells were incubated with 50 μM contrast agents for an initial 24 hr absorption period. The media containing the contrast agents were then removed, followed by three PBS washes, and the cells were then allowed to leach the intracellular portion of the contrast agent into serum-free media for 1, 2, 4, 6, 24, or 48 hr. The amount of the contrast agents was then determined by quantifying the amount of compound inside the cell using ICP-MS and then divided by the amount of gadolinium in the leached media minus the background (Figure 3C). Although **1** did not appear to leach from the MDA-MB-231 cells more quickly, compound **2** showed much higher retention in the T47D cell line as compared to MDA-MB-231. Therefore, one way that these compounds may specifically mark progesterone receptor-positive cells is by residing in the cell longer, due to interaction with PR.

Steroid Conjugates Function to Transcriptionally Upregulate PRE-Luciferase

Progesterone receptors bind to a region of DNA referred to as the progesterone response element (PRE). This DNA element (repeated three times) was ligated to DNA encoding the luciferase gene and used to (1) monitor the cell permeability of the compounds, (2) verify the ability to interact with the progesterone receptor dimer, and (3) evaluate function in a transcription complex (Figure 4). For the progesterone derivatives, induction of luciferase indicated that the compound was functional. Each compound showed the ability to alter transcription of the PRE, indicating that it entered the cell and bound to the full-length progesterone receptor. Agent **2** proved the most effective transcriptional agent and differed marginally from compound **3** in its ability to bind to the receptor. This indicates that a neutral charge on the agent is beneficial for activity and permeability. A hydrophobic linker between the progesterone molecule and the Gd(III) chelate appeared to improve cell permeability and may increase transcription, because the compounds are inside the cell interacting with the progesterone receptor for a longer period of time. These data confirm that although compound **2** does not bind as readily to the PR as compound **1**, the improved cell permeability makes the agent enter the cells more rapidly, at lower doses, and provides higher transcriptional activity.

Steroid-Based Contrast Agents Enhance T_1 Relaxivity In Vitro

To determine the effect of **1** and **2** on T_1 after receptor binding, we obtained T_1-weighted images and measured spin-lattice relaxation times of incubated cells (Figure 5A). Compound **1** was chosen because of its high binding affinity and compound **2** because of efficient cellular uptake. Progesterone receptor-positive cells (T47D, MDA-MB-231 transfected with PRA) and -negative cells (MDA-MB-231) were incubated with 50, 150, and 500 μM **1** and **2** for 24 hr prior to scanning.

T_1-weighted images and relaxation times show that compound **2** enhanced MR contrast significantly more than compound **1** in any given cell type. All cells that were treated with compound **2** appeared much brighter than the cells that were treated with compound **1** or control media. For example, cells exposed to 150 μM **2** reduced T_1 more than 60% compared to controls. There are no significant changes in T_1 at 500 μM **2**, indicating

intracellular Gd(III) was quantified using ICP-MS. Data represent the average of triplicate determinations divided by the amount of cellular protein to normalize content based on cell number.

(B) Progesterone Gd(III) chelates are time dependently absorbed into mammalian breast cancer cells with and without progesterone receptors. Cells were incubated with 50 μM contrast agents for 1, 2, 4, or 24 hr, washed with PBS, and lysed, and the amount of intracellular Gd(III) was quantified using ICP-MS. Data represent the average of triplicate determinations divided by the amount of cellular protein to normalize content based on cell number.

(C) Progesterone gadolinium chelates are selectively retained in progesterone receptor-expressing cells at specific time points after leaching into culture medium. Cells were incubated with 50 μM contrast agents for 24 hr, washed with PBS at 0.5, 1, 2, 4, 6, 24, and 48 hr, lysed, and the amount of intracellular and effluxed Gd(III) was quantified using ICP-MS. Data represent the average of triplicate determinations with the amount of Gd(III) in the cell divided by the amount in the media and then divided by the amount of cellular protein to normalize content based on cell number (x axis, incubation time; y axis, retention ratio).

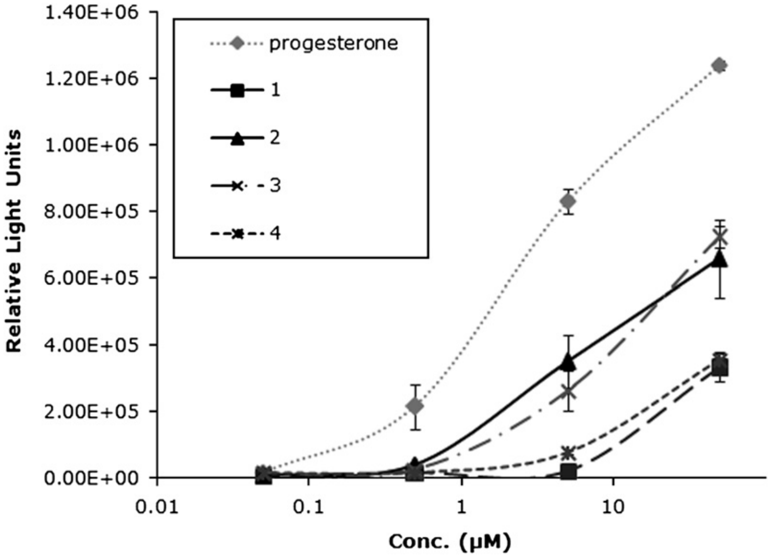


Figure 4. Progesterone Gd(III) Chelates Function Biologically to Initiate Gene Transcription of a Progesterone Responsive Element

T47D cells were transiently transfected with the 3×PRE-luciferase construct and serum starved for 24 hr before treatment with contrast agents for an additional 24 hr. Relative light units were calculated and the data represent the average ± the standard deviation from triplicate measurements.

that the cells were saturated with compound **2** at 150 μM and no further uptake occurred.

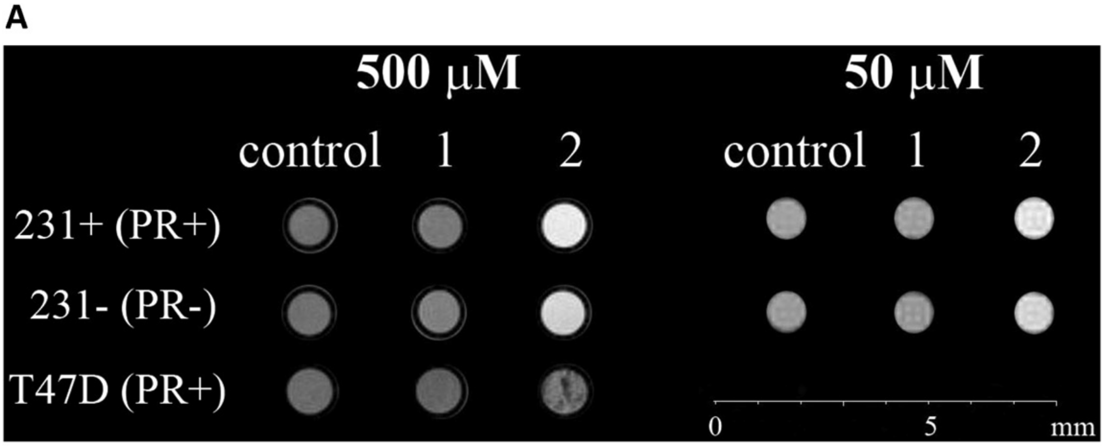
DISCUSSION

A flaw with currently available magnetic resonance contrast agents is their lack of intracellular accumulation, limiting their specificity to extracellular compartments. Proteins that reside inside the cell offer additional targets for specificity. For hormone-dependent cancers, the expression of progesterone receptor is one such target and the conjugation of the gadolinium complex allows for cell penetration. In this study, several novel MR agents were synthesized and evaluated to target progesterone receptors. The most promising agent, compound **2**, demonstrated hormone receptor binding, progesterone-responsive gene transcription, and enhanced intracellular relaxivity. Our results indicate that steroid receptor-specific MR agents can be prepared and retain their ability to interact with their receptor to enhance relaxivity.

The progesterone-MR contrast agent conjugates were designed to optimize receptor interaction. A series of agents was synthesized with variable linkers between the Gd(III) chelate and the hormone backbone. The impact of linker length on receptor interaction was examined using progesterone receptor binding experiments and surprisingly demonstrated that the agents with the highest affinity were those that had no spacer between the chelate and hormone, such as compound **1**. Because it has a hydrophilic Gd(III) chelate instead of having a lipophilic chain on the 21 position, this might indicate that there is a favorable interaction between receptor protein and the chelate. Altering the carbon chain with a hydrophilic PEG linker or poly-amino acid chain may result in higher receptor binding affinity. The progesterone Gd(III) chelates had an approximately 100-fold higher affinity for this receptor than the chelate conjugated to RU-486 [23]. Incorporating a linker region between the steroid and the chelate resulted in lower binding affinity for the receptor, perhaps through disruption of dimer formation. The relatively tight binding of the hormone with the receptor demonstrates that the modification of the steroid decreases but does not prohibit interaction with the progesterone receptors.

Conjugation of a Gd(III) chelate to the steroid progesterone allowed for significant and rapid cellular accumulation. All of the compounds traversed the cell membrane in a dose-dependent manner. Conjugate **2** most readily entered the cells, as indicated by ICP-MS and X-ray analysis. Compound **1**, however, was not as membrane permeable as the compounds containing a six-carbon extended spacer, likely due to its relatively low lipophilicity. The addition of different charges on the chelate adversely affected cell permeability, probably due to the inability of the charged group to readily pass the hydrophobic membrane. These experiments demonstrate that the steroid conjugation of the Gd(III) chelate allows it to be carried into the cell where it may interact with the progesterone receptor.

Progesterone-conjugated Gd(III) chelates are cell permeable and interact with the progesterone receptor, suggesting they could provide cell-specific image enhancement. Activation of the specific biological target by the contrast agent was directly demonstrated using the transcriptional activation of the PRE-luciferase construct. Transcriptional activation is important for specific cellular targeting because the contrast agent would be engaged in a receptor:DNA complex and might be retained within the cell. By directly comparing the amount of gadolinium that leached from progesterone receptor-expressing T47D cells as compared to receptor-negative MDA-MB-231 cells, a relative retention was calculated. Compound **2** was specifically retained in receptor-expressing cells. Compound **1** was not specifically retained, likely because the initial absorption of the compound is relatively low. Cellular retention is likely different between **1** and **2**, due to the enhanced interaction with the receptor after high



B

T_1 **at 50 μM (ms)**

	Control	1	2
231+ (PR+)	3720 ± 200.5	3774 ± 239.5	2481 ± 124.0
231- (PR-)	3802 ± 251.9	3922 ± 261.0	2739 ± 143.0
T47D (PR+)	n/d	n/d	n/d

T_1 **at 150 μM (ms)**

	Control	1	2
231+ (PR+)	3473 ± 232.0	2701 ± 193.9	1521 ± 116.2
231- (PR-)	3616 ± 263.6	2737 ± 195.3	1514 ± 114.6
T47D (PR+)	3310 ± 207.9	2849 ± 218.9	1742 ± 154.5

T_1 **at 500 μM (ms)**

	Control	1	2
231+ (PR+)	3530 ± 4.122	3530 ± 30.75	1743 ± 14.60
231- (PR-)	3528 ± 57.90	3482 ± 47.81	1813 ± 27.22
T47D (PR+)	3440 ± 27.79	n/d	n/d

Figure 5. In Vitro MRI Results

(A) T_1-weighted images of breast cancer cells incubated with compounds **1** and **2** for 24 hr. Gd(III) chelates demonstrate enhanced cell relaxivity in vitro. The images were obtained at 9.4 T, field of view = 1.5 cm, T_E = 10 ms, T_R = 200 ms (50 μM), T_E = 10.2 ms, T_R– 600 ms (500 μM).

(B) T_1 data of cells incubated with 50, 150, and 500 μM **1** and **2** (T_1 data were taken at 9.4 T, 20°C).

absorption and the generation of a transcription complex. Evidence of transcriptional regulation is supported by the high level of luciferase activity generated by compound **2** in the PRE transcription assay. The conjugate-induced transcription provides evidence of cellular permeability, receptor-mediated retention, and a lack of toxicity. Cellular transcription suggests that these contrast agents may be useful for obtaining images long after traditional agents that are readily excreted. Future studies must be completed to investigate the cell-specific retention of these contrast agents in vivo, where the effects of metabolism and tissue distribution will critically determine cell specificity.

Progesterone-based contrast agents provide magnetic resonance signal enhancement inside breast cancer cells. The T_1 effects of compound **2** were substantially changed at 150 μM in MDA-MB-231 cells both with and without receptor. Incubating the cells with higher doses of the contrast agent did not produce additional signal enhancement. Compound **1** was expected to produce a greater τ_r effect because it has the highest binding affinity for the receptor and the slowest rotation of the Gd(III) chelate due to the absence of the six-carbon spacer. However, membrane permeability of **1** was significantly lower even at the highest concentration (500 μM) and failed to enhance MR contrast in vitro. Compound **2** showed high cellular accumulation; however, differences in the amount of accumulation inside cells between PR-positive and PR-negative were insignificant, thereby creating MR images with the same signal intensity. The changes in T_1 using relatively low doses of contrast agent indicate that these newly synthesized compounds are viable contrast agents that can be utilized in low doses.

In conclusion, a series of progesterone-modified Gd(III) chelate conjugates was synthesized to generate contrast agents that accumulate intracellularly and interact with a biological target highly relevant for cancer prognosis. The chelates varied in linker length and charge, and we have identified compound **2** as the most efficient agent for progesterone receptor binding and intracellular accumulation. The aliphatic carbon linker between the steroid and the Gd(III) chelate dramatically improved cellular permeability while retaining transcriptional activation of the progesterone responsive element without toxicity. Our results show that changing the modification site from 3-keto to 21-hydroxyl enhanced the binding affinity approximately 100-fold as compared to a previously reported RU-486-modified contrast agent [23]. The observed relaxivity of the contrast agent in mammary cells was significant.

SIGNIFICANCE

Noninvasive means of determining the steroid receptor profile of breast cancers will improve diagnosis and treatment of disease. An ideal imaging agent will enter the cell, interact with its biological target, and specifically increase relaxivity to allow for proper MR image acquisition. The first generation of progesterone-based gadolinium contrast agents was developed to image hormone-responsive tumors using MRI by taking advantage of the interaction intracellularly with the receptor. A novel chemical synthesis approach was utilized to develop progesterone-modified gadolinium agents. In this study, progesterone gadolinium contrast agents were characterized and found to interact with progesterone receptors, enter breast cancer cells, and significantly enhance MRI contrast. Therefore, future studies will determine whether these agents will be useful for specifically marking hormone receptor-expressing cells in both in vitro and in vivo animal systems. The contrast agents present the opportunity to image the location and hormone status of breast tumors.

EXPERIMENTAL PROCEDURES

Synthesis

Compounds **1**–**4** were synthesized by following the pathway described in Figure 2. Detailed synthetic procedures and spectroscopic data are available in the Supplemental Data.

Octanol-Water Partition Coefficient Measurements

Octanol-water partition coefficients were obtained by dissolving 5–7 mg of each compound (**1**–**4**) into mixtures of 500 μl water and 500 μl 1-octanol. The resulting mixture was shaken vigorously for 2 hr on a Lab-Line lab rotator (model 1304; Lab-Line, Dubuque, IA, USA). The solvent layers were allowed to separate, and 400 μl of each layer was removed. The solvent was removed under reduced pressure, and the mass of material from each layer was measured. The reported values are for the mass of compound in the 1-octanol layer divided by the mass of compound in the water layer. Octanol-water partition coefficient measurements were repeated in triplicate.

Relaxivity Measurements

Relaxivity measurements were acquired by taking the slope of a plot of T_1^{-1} versus concentration. The longitudinal water proton relaxation time (T_1) at 59.97 MHz was measured using a Bruker mq60 NMR analyzer (Bruker Canada, Milton, ON, Canada). A 4 mM stock solution of each compound in deionized water was diluted to give 500 μl each of six concentrations for each run: 0, 0.125, 0.25, 0.5, 1.0, and 2.0 mM. For **3**, a 1 mM stock solution in deionized water was diluted to give 250 μl each of six concentrations for each run: 0, 0.001, 0.005, 0.01, 0.05, and 0.1 mM. The T_1 of each concentration was determined using an inversion recovery pulse sequence with appropriate recycle delays. The resulting curves were fit to a monoexponential function to obtain T_1. All lines fit with $r^2 > 0.998$, and the measurements were performed in duplicate.

Progesterone Receptor Binding Assay

The progesterone receptor A ligand binding domain (amino acids 675–933) fused to GST (PR-LBD-GST; 80 nM), a fluorescently tagged PR ligand (fluoromone green PL; 4 nM), and either progesterone (1 μM) or compound **1**–**4** (several concentrations) were incubated in PR screening buffer with 4 mM dithiothreitol (DTT) in a total volume of 100 μl for 1 hr at room temperature according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). Each sample was performed in at least triplicate using the Beacon 2000 fluorescence polarization analyzer (Invitrogen) located in the Northwestern University Keck Facility. The machine was used in static mode, batch blank, no delay, with an average of 1 read per cycle, at 22°C. An average of three samples containing only buffer and PR-LBD-GST with no fluorescent PL was used as the blank to eliminate background signal from the protein or buffer. A sample with no competitor was used to determine 100% binding capacity of the PR-LBD-GST for the PL ligand.

Progesterone Response Element-Luciferase Transcriptional Activation

T47D breast cancer epithelial cells (American Type Culture Collection, Manassas, VA, USA) were cultured in phenol red-free RPMI (Life Technologies, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) and 1% antimycotic/antibiotic (Invitrogen) and incubated at 37°C, under 5% CO_2. Cells were plated 1 day before transfection in 24-well plates and transiently transfected in Opti-MEM (Invitrogen) with PRE-luciferase (a kind gift from Ken Korach, NIEHS, NIH). Cells were then treated with serum-free media and vehicle (DMSO), progesterone, and progesterone-modified contrast agents for 24 hr. To measure luciferase production, cells were lysed in GME buffer (25 mM glycylglycine [pH 7.8], 15 mM $MgSO_4$, 4 mM EGTA, 1 mM DTT, and 1% Triton X-100) and lysates were added to assay buffer (GME buffer, 16.5 mM KPO_4, 2.2 mM ATP, and 1.1 mM DTT). Luciferase activity was measured for 30 s using an AutoLumat (Berthold Technologies, Oak Ridge, TN, USA). A separate protein determination using the BCA kit (Pierce, Rockford, IL, USA) was used to normalize protein levels that might differ from treatment with hormone.

Mammary Cell Progesterone-Gd(III) Cellular Uptake

Progesterone receptor-positive cells, T47D, and progesterone receptor-negative cells (MDA-MB-231) were used to determine uptake efficiency of progesterone-modified contrast agents into hormone receptor-expressing cells. MDA-MB-231 breast cancer epithelial cells (American Type Culture Collection) were cultured in phenol red-free DMEM/F12 (Life Technologies) supplemented with 10 μg/ml insulin, 5% charcoal dextran-stripped FBS (Cellgro, Herndon, VA, USA), 1% glutamax, and 1% antimycotic/antibiotic (Invitrogen) and incubated at 37°C, under 5% CO_2. Cells were plated into 12-well dishes and the next day were moved into serum-free media for 24 hr before treatment with compound **1**–**4**. To determine the dose of PR-Gd necessary to accumulate in the cells, the following doses were incubated with cells for 24 hr: 0, 0.05, 0.5, 5, and 50 μM. In order to determine the optimal incubation time, 50 μM PR-Gd compounds was incubated with the cells for the following time periods: 0, 1, 2, 4, and 24 hr. Data were analyzed by counting the cells, followed by lysis and ICP-MS. For leaching experiments, contrast agents were incubated with the cells for 4 hr, removed, and rinsed with PBS. At each time point after the initial rinse, cell media were removed, rinsed, and replaced with serum-free media and then collected at 15 min, 1, 2, 4, 6, 24, 48, and 72 hr. The cells were also collected by trypsinization and lysed to compare intracellular content of the cells with that which leached into the media.

Synchrotron Radiation X-Ray Fluorescence Analysis

The receptor-positive and -negative cells were incubated with **1** and **2** for 24 hr prior to analysis. The cells were washed and collected by following the same procedure as described above. Approximately 15 μl of each suspension was applied to Formvar-coated gold grids with a sterile glass Pasteur pipette for 1 min and the excess supernatant was removed. This was followed by addition of approximately 15 μl of room temperature ethanol, removal of the ethanol, and drying at ambient temperature for 15 hr. The cell coverage was approximately 15–30 cells/grid. Electron microscope grids with cells were mounted onto a kinematic specimen mount for both visible light and X-ray fluorescence microscopy. The samples were examined under a light microscope (Leica DMXRE, Solms, Germany), and the cells to be scanned with SR-XRF were placed on the grid relative to a reference point using a high spatial resolution motorized x/y stage (Ludl Bioprecision, Hawthorne, NY, USA).

X-Ray Fluorescence Microscopy

Synchrotron scanning X-ray fluorescence microscopy was carried out at the 2-ID-E beamline of the Advanced Photon Source at Argonne National Laboratory (Argonne, IL, USA). Hard X-rays (10 keV) from an undulator source were monochromatized using a single-bounce Si < 111 > monochromator. The energy was selected to allow for efficient excitation of the Gd L lines and to enable the detection of the Zn K lines. A Fresnel zone plate (320 μm diameter, focal length f = 250 mm, Xradia, Concord, CA, USA) was used to focus the monochromatic X-ray beam to a spot size of approximately $0.3 \times 0.3\ \mu m^2$ on the specimen. The sample was raster scanned through the beam at room temperature under a helium atmosphere. At each scan position, a full fluorescence spectrum was acquired using an energy dispersive germanium detector (Ultra-LEGe; Canberra, Meriden, CT, USA). Elemental content was determined by comparison of fitted sample spectra with National Bureau of Standards thin film standards 1832 and 1833 (National Institute of Standards and Technology, Gaithersburg, MD, USA) using MAPS software supplemented with fitting of fluorescence spectra at each pixel.

T_1-Weighted Image Acquisition

The receptor-positive cells (T47D and MDA-MB-231 transfected with PRA) and -negative cells (MDA-MB-231) were incubated with no agent or 50, 150, and 500 μM compound **1** and **2** for 24 hr at 37°C. Cells were loaded into capillary tubes (1 mm diameter) as trypsin suspensions. All MR data were collected at ambient temperature in a General Electric/Bruker Omega 400WB 9.4 T magnet (83 mm bore size) fitted with Accustar shielded gradient coils (Bruker, Westmont, IL, USA). Spin-lattice relaxation times (T_1) were measured using an inversion recovery pulse sequence, and images were acquired using a T_1-weighted spin-echo pulse sequence with a repetition time (T_R) of 100∼2000 ms and an echo time (T_E) of 10∼10.2 ms.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures and one figure and can be found with this article online at http://www.chembiol.com/cgi/content/full/14/7/824/DC1/.

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