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PAMAM Dendrimer-Based Contrast Agents for MR Imaging of Her-2/*neu* Receptors by a Three-Step Pretargeting Approach

Wenlian Zhu, Baasil Okollie, Zaver M. Bhujwalla, and Dmitri Artemov*

JHU ICMIC Program, Russell H. Morgan Department of Radiology and Radiological Science, Johns Hopkins University School of Medicine, Baltimore, Maryland.

Abstract

Pretargeting of receptors is a useful approach in molecular imaging and therapy to reduce background noise or toxicity and enhance selectivity. In this study a three-step pretargeting approach that includes a biotinylated antibody, avidin/streptavidin, and a biotinylated imaging agent is described. A PAMAM dendrimer generation 4 (G4D)-based MRI T_1 agent biotin-G4DD-TPA-Gd (bG4D-Gd) and its sister compound with remaining free surface amine groups blocked by succinic anhydride to reduce positive charges (bG4D-Gd-SA) were synthesized. Limited selective enhancement in MRI was observed in a Her-2/*neu* mouse tumor xenograft by this three-step pretargeting approach that includes biotinylated trastuzumab, avidin and bG4D-Gd, or bG4D-Gd-SA. However, these dendrimer-based MRI agents with molecular weight around 29 kD reached and remained in the tumor through the enhanced permeability and retention effect. Prolonged and extensive accumulation of both bG4D-Gd and b-G4-Gd-SA in the kidneys was also observed.

Keywords

pretargeting; Her-2/*neu* receptors; PAMAM dendrimer; MRI

A three-step pretargeting approach pioneered by Paganelli et al. (1) about 20 years ago using biotin-avidin systems still holds promise in increasing the tumor/nontumor ratio compared to a direct targeting approach (2). While more popular in the delivery of radionuclides for imaging and therapy, the low concentration of receptors and the intrinsic low sensitivity of MRI have limited applications of pretargeting in MRI. Her-2/*neu* overexpressing breast tumors are a good model system for an MRI pretargeting approach due to the large number of receptors expressed uniformly on the cancer cell surface and the availability of trastuzumab, an FDA-approved antibody against the Her-2/*neu* receptors (3,4). Success in delivering MRI agents by pretargeting can also be extended to therapy, which is important as the amplification and overexpression of Her-2/*neu*, a member of the epidermal growth factor receptor (EGFR) family of tyrosine kinases that regulates cell proliferation and differentiation, has been linked to poor prognosis in several human cancers such as breast, prostate, lung, ovary, and colon cancers.

Because of its low intrinsic sensitivity, the success of MRI depends on the development of highly efficient contrast agents. For example, it was demonstrated that conjugating of Gd(III) chelates to high molecular weight carriers is highly beneficial for in vivo MR imaging as it

provides 1) prolonged intravascular circulation time that results in improved pharmacokinetics of the agent at the target site, and 2) slows down molecular rotation that translates to increased T_1 relaxivity at high magnetic field typically used in preclinical MRI studies (5). Macromolecular MRI contrast agents based on PAMAM dendrimers conjugated with a diethylenetriaminepentaacetic acid (DTPA) derivative, 2-(p-isothiocyanatobenzyl)-6-methyl-diethylenetriaminepentaacetic acid (1B4M), to chelate gadolinium metal ions have been proven to be versatile in MRI applications due to their mono-disperse molecular size and the ability to fine-tune molecular weights by using dendrimers of different generations (6–8). In our studies we chose a PAMAM generation 4 dendrimer (G4D) due to its i) 64 surface functional amine groups ($-\text{NH}_2$) that can be easily modified, ii) moderate blood circulation time, and iii) relatively fast excretion via the kidney (7). It was reported that a G4-based gadolinium agent was quickly excreted via the kidney primarily during the first pass, while exhibiting no measurable leakage from normal blood vessels (6).

The surface amine groups of PAMAM dendrimers are positively charged at physiological pH. Microvascular endothelium is lined with a glycocalyx layer which is composed of negatively charged sulfated glycosaminoglycans (9,10). Electric static interaction between the positive amine group and the negative endothelium promotes faster clearance as well as nonspecific binding of the PAMAM dendrimer. Cationic macromolecules also show higher glomerular permeation than anionic macromolecules of similar molecular weights, as glomerular capillary walls function as a charge selective barrier having negative charge (11). Conjugation of each G4D $-\text{NH}_2$ group to diethylenetriaminepentaacetic acid (DTPA) chelating gadolinium(III) (DTPA-Gd) resulted in a net charge of -1 , helping to reduce the overall positive charge of the final complex $\text{G4D}-(\text{DTPA-Gd})_n$. Local positive charges due to the remaining free surface amine groups that were not conjugated to (DTPA-GD) can be further reduced by succinylation with succinic anhydride.

In the present study, athymic mice bearing Her-2/*neu* overexpressing human breast tumor BT-474 xenografts were imaged through a three-step pretargeting approach that included biotinylated trastuzumab, avidin, and bG4D-Gd. To test the effect of the surface charge of bG4D-Gd on blood circulation time and kidney excretion we also used its succinylated form, bG4D-Gd-SA.

MATERIALS AND METHODS

Materials

Human breast cancer BT-474 and MCF-7 cells and cell medium 46-X were obtained from American Type Culture Collection (Manassas, VA). The β -estradiol pellets were obtained from Innovative Research of America (Sarasota, FL). Trastuzumab was obtained from Genentech (South San Francisco, CA). Sulfo-NHS-LC-Biotin and HABA reagents were obtained from Pierce (Rockford, IL); 50% deglycosylated avidin lite was obtained from Accurate Chemical (Westbury, NY). PAMAM dendrimer G4D (Sigma 412449), Eagle's minimum essential medium (EMEM), DTPA dianhydride, acetic anhydride, and succinic anhydride were obtained from Sigma (St. Louis, MO). Alexa Fluor594 carboxylic acid succinimidyl ester (A-20004) was obtained from Molecular Probes (Eugene, OR). Athymic mice (female, 4–6 weeks old, ≈ 30 g) were purchased from NCI (Bethesda, MD).

Tumor Model

Human breast cancer BT-474 cells were grown in 46-X medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin and maintained at 37°C in 5% CO_2 . Human breast cancer MCF-7 cells were grown in EMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin and maintained at 37°C , in 5% CO_2 . For BT-474 tumor models,

athymic mice were inoculated in a thoracic mammary fat pad with 1×10^7 BT-474 cells mixed with an equal volume of Matrigel, 24–48 hr following the subcutaneous implantation of a 60-day release 0.72 mg β -estradiol pellet. For bilateral tumor models, athymic mice were inoculated in a left thoracic mammary fat pad with 1×10^7 BT-474 cells mixed with equal volume of Matrigel, and a right thoracic mammary fat pad with 3×10^6 MCF-7 cells, 24–48 hr following the subcutaneous implantation of a 60-day release 0.72 mg β -estradiol pellet. Tumors were grown to an average volume of 0.25 cm^3 . All animal experiments were performed in accordance with institutional guidelines.

Conjugation

Trastuzumab and G4D was biotinylated with Sulfo-NHS-LC-Biotin following the manufacturer's (Pierce) protocol. After the purification by ultrafiltration with an Amicon Ultra-15 Centrifugal Filter Unit with a 10 kDa membrane from Millipore (Billerica, MA), the final biotin/trastuzumab or biotin/G4D ratio was ≈ 4 as determined by the HABA method (Pierce). Biotinylated G4D were further conjugated to DTPA and gadolinium to produce bG4D-Gd as described (3). For succinylation, about 60 mg of succinic anhydride was added to 46 mg bG4D-Gd dissolved in 25 mL of 50 mM sodium bicarbonate buffer at pH 9; pH was maintained at 7 during the addition of succinic anhydride with 1 M NaOH. The succinylation reaction proceeded for 1.5 hr at room temperature and the final product bG4D-Gd-SA was purified through ultrafiltration.

For optical studies of the cells the biotinylated G4D was conjugated to Alexa Fluor594 following the manufacturer's (Invitrogen, La Jolla, CA) protocol. The free amines of this product were further blocked with acetic anhydride.

Fluorescence Studies of BT-474 Cells

Approximately 2×10^4 BT-474 cells were seeded per chamber on Lab-Tek II four chamber slides (Nalge Nunc International, Rochester, NY). Cells were treated with biotinylated trastuzumab at $30 \mu\text{g/mL}$ for 10 min, washed, treated with streptavidin-Alexa488 (Invitrogen) at $4 \mu\text{g/mL}$ for 5 min. Cells were subsequently washed and treated with biotinylated G4D-Alexa594 conjugate at $4 \mu\text{g/mL}$ for 10 min. Cells were then washed and fixed with 3% paraformaldehyde in phosphate-buffered saline (PBS) before being imaged with a Nikon Eclipse E400 Microscope; a $40\times$ objective was used.

MRI Studies of Mice by Three-Step Pretargeting Approach

BT-474 tumor-bearing mice received 1 mg biotinylated trastuzumab (nonbiotinylated was used as control) intravenously 48 hr before the administration of 5 mg avidin and 6 mg of bG4D-Gd or bG4D-Gd-SA (0.145 mmolGd/kg) were injected 2 hr later. Mice were anesthetized with an intraperitoneally (i.p.) injection of a ketamine/acepromazine mixture (25 and 2.5 mg/kg, respectively, in saline) before the MRI studies. Body temperature of the mice was maintained at 37°C by heat generated from a pad circulating with warm water placed under the MRI probe. MR studies were performed on a 9.4T Bruker Biospec spectrometer. Quantitative T_1 MR images were obtained by a saturation-recovery multislice spin-echo pulse sequence. Saturation-recovery T_1 images of three or four slices (slice thickness of 2 mm) were acquired with six relaxation delays of 250 ms, 500 ms, 1 sec, 2 sec, 4 sec, and 8 sec with an in-plane spatial resolution of 0.250 mm (128×64 matrix zero-filled to 128×128 , field of view = 32 mm, number of scans = 8). Quantitative T_1 relaxation maps were reconstructed from datasets for six different relaxation times using the IDL program. The same three-step pretargeting MRI experiments were performed on BT-474 and MCF-7 bilateral tumors bearing athymic mice. MRI studies were also conducted as above on a BT-474 tumor-bearing mouse that received a reduced dose of 3 mg of bG4D-Gd (0.072 mmolGd/kg) only.

Molecular Weight Determination

The molecular weight of bG4D-Gd was determined by Global Peptide (Fort Collins, CO) with MALDI-TOF MASS spectrometry. The molecular weight of both bG4D-Gd and b-G4-Gd-SA were further confirmed at the Mass Spectrometry/Proteomics Facility at the Johns Hopkins University School of Medicine.

RESULTS

Conjugation of Biotinylated G4D to DTPA-Gd

The molecular weight of bG4D-Gd was about 29 kD as determined by MALDI-TOF MASS spectrometry, which gave us an approximate formula of biotin₄-G4D-(DTPA-Gd)₂₁, which means that an average of 21 out of 64 G4D surface -NH₂ groups were successfully conjugated to (DTPA-Gd) and the gadolinium weight ratio was 14.6%. The relaxivity of this compound was determined at room temperature on a 9.4T Bruker Biospec spectrometer to be 137 mM⁻¹s⁻¹.

Succinylation of bG4D-Gd

Succinylation with succinic anhydride of biotin₄-G4D-(DTPA-Gd)₂₁ increased the molecular weight of the final product by about 1500 Da, indicating partial blocking (15) of the remaining 43 free -NH₂ surface groups of bG4D-Gd due to incomplete (DTPA-Gd) conjugation.

Treatment of BT-474 Cells Followed by the Three-Step Pretargeting Approach

Microscope images of BT-474 cells labeled with biotinylated trastuzumab, streptavidin-Alexa488, and biotinylated G4D-Alexa594 are shown in Fig. 1. Streptavidin-Alexa488 produced strong membrane staining of the BT-474 cells, indicating successful binding of streptavidin-Alexa488 to biotinylated trastuzumab. However, the staining from biotinylated G4D-Alexa594 conjugate was much weaker, which may be a result of the limited binding of biotinylated G4D-Alexa594 to streptavidin-Alexa488.

MRI Enhancement of bG4D-Gd Followed by the Three-Step Pretargeting Approach

Axial *T*₁-weighted MR images of nude mice bearing BT-474 tumors treated with biotinylated trastuzumab (nonbiotinylated trastuzumab was used as control), avidin, and bG4D-Gd are shown in Fig. 2. Precontrast and 24-hr postcontrast *T*₁ images are shown for comparison. At 24 hr a significant amount of bG4D-Gd accumulated in the treated mice. Tumor tissue showed stronger enhancement when compared to muscle tissue. Coronal images (Fig. 3) clearly show the contrast generated by bG4D-Gd at 24 hr. While the overall signal intensity is increased, the enhancement is mostly concentrated in the internal organs. Following a three-step pretargeting approach, the mouse treated with biotinylated trastuzumab did not show significantly lower tumor *T*₁ than the mouse treated with trastuzumab (Table 1).

MRI Enhancement of bG4D-Gd-SA by the Three-Step Pretargeting Approach

Axial *T*₁ MR images of nude mice bearing BT-474 tumors treated with biotinylated trastuzumab (nonbiotinylated trastuzumab was used as control), avidin, and bG4D-Gd-SA are shown in Fig. 4. Precontrast and 24-hr postcontrast *T*₁ images are shown for comparison. bG4D-Gd-SA showed similar contrast enhancement in the tumor to that of bG4D-Gd in a three-step approach. Mouse treated with a three-step targeting approach with biotinylated trastuzumab did not show lower tumor *T*₁ when compared to that of the nontargeting approach with trastuzumab.

MRI Enhancement of bG4D-Gd

Axial T_1 MR images of a nude mouse bearing a BT-474 tumor treated with bG4D-Gd only is shown in Fig. 5. Observing the strong overall MRI enhancement produced by both bG4D-Gd and bG4D-Gd-SA, the amount of bG4D-Gd given to this mouse was reduced from 6 mg to 3 mg. At 15 min after the injection of bG4D-Gd the entire kidneys were brightened by this agent while the tumor was moderately enhanced. At 24 hr the enhancement of the tumor due to bG4D-Gd persisted, while the kidney enhancement was mostly concentrated in the outer cortex.

MRI Enhancement of bG4D-Gd by the Three-Step Pretargeting Approach in a Bilateral Tumor Model

To directly compare the effectiveness of our three-step pretargeting approach that includes biotinylated trastuzumab, avidin, and bG4D-Gd, we used a bilateral tumor model that included an MCF-7 tumor with low Her-2/*neu* expression and a BT-474 tumor with high Her-2/*neu* expression (4). Four athymic mice bearing such bilateral tumors were treated with 1 mg biotinylated herceptin, 6 mg avidin, and 3 mg bG4D-Gd. At 24 hr after the administration of the contrast both MCF-7 and BT-474 tumors showed comparable T_1 values, as shown in Table 2.

Long-Term Retention of bG4D-Gd-SA

Both axial and coronal MR images taken 101 days after the injection of 6 mg bG4D-Gd-SA show that a minor residue of the contrast agent still remained in the mouse, mainly in the kidney outer cortex, as displayed in Fig. 6. Images taken 30 days after the injection of 3 mg bG4D-Gd also showed similar contrast residue in the outer cortex of the kidney.

DISCUSSION

Our results showed that with bG4D-Gd alone, the targeting three-step approach with biotinylated trastuzumab, avidin, and bG4D-Gd or bG4D-Gd-SA, and the nontargeting three-step approach with trastuzumab, avidin, and bG4D-Gd or bG4D-Gd-SA produced similar T_1 enhancement of the tumor tissues, as shown in Table 1. Following the three-step pretargeting approach, MCF-7 and BT-474 bilateral tumor-bearing mice displayed comparable gadolinium accumulation in both tumors with different Her-2/*neu* receptor expression levels. These can be the consequence of the inherent enhanced permeability and retention (EPR) of macromolecules due to tumor vasculature (12) and an ineffective biotin-avidin recognition system in the three-step pretargeting approach.

Being macromolecular contrast agents of molecular weight around 29 kD, PAMAM dendrimer G4-based Gd complexes are capable of accumulating in the tumor tissues due to the EPR property of the tumor vasculatures. This is a surprise, as previously it was suggested that EPR only applied to macromolecules larger than 45 kD (13). However, it was also reported that a PAMAM generation 3.5-platinate was able to selectively increase the platinum content of the tumor 50-fold compared to that of cisplatin due to the EPR effect (14). Our results showed that 3 mg of bG4D-Gd alone produced an observable T_1 decrease at the tumor site 24 hr after the injection. The EPR effect, especially the retention of macromolecular agents by tumor tissues, renders a three-step pretargeting approach with an antibody less relevant when macromolecules are used as a carrier of the imaging or therapeutic agents. A three-step pretargeting approach is only meaningful when the tumor actively retains the targeting antibody only. The other two components must be able to freely diffuse in and out of the tumor vasculature quickly. They should only be retained in the tumor tissues when they are attached to the antibody through a stable bridge such as a biotin avidin recognition system in order to maintain antibody selectivity.

Avidin/streptavidin is a tetramer that can bind four biotin molecules with high affinity. It was because of this strong multiple biotin binding ability that biotin-avidin/streptavidin was used as a bridge in a three-step pretargeting approach. In this approach the biotinylated effector is expected to bridge to the biotinylated antibody through avidin/streptavidin, as shown in Fig. 7. However, in reality avidin/streptavidin will bind to the available biotinylated antibody first before the addition of the biotinylated effector. It is possible that in extreme cases biotinylated antibody alone can saturate all four binding sites of avidin/streptavidin and leave no room for the biotinylated effector, as depicted in Fig. 7. Our three-step BT-474 cell labeling results showed that biotinylated G4D-Alexa594 was still able to bind to the streptavidin-Alexa488/biotinylated trastuzumab complex, although with much reduced intensity, as shown in Fig. 1, which could be the result of partial saturation of streptavidin by biotinylated trastuzumab. MRI studies of mice treated with biotinylated trastuzumab, avidin, and bG4-Gd (or bG4-Gd-SA) showed no significantly stronger tumor T_1 enhancement than those treated with trastuzumab, avidin, and bG4-Gd (or bG4-Gd-SA). This was most likely due to the saturation of avidin binding sites by biotinylated trastuzumab and, subsequently, the binding of bG4-Gd (or bG4-Gd-SA) to the biotinylated trastuzumab/avidin complex was mostly blocked. Therefore, a three-step pretargeting approach failed to produce selective tumor enhancement. The comparable T_1 enhancement at the tumor site from the three-step targeting or nontargeting approach was due to the enhanced permeability of the tumor vasculature and retention of bG4-Gd or bG4-Gd-SA within the interstitium.

The three-step pretargeting approach was based on the two-step biotin-avidin approach to fully exploit the rapid pharmacokinetics of biotin (1,15). Additionally, a clearance step by avidin may be used to rapidly transport free biotinylated antibody into the liver. We previously reported that the application of an avidin/streptavidin bridge system resulted in the cross-linking of the biotinylated antibody at the tumor cell surface. This cross-linking subsequently leads to the accelerated internalization of the antibody (16). With proper timing, it may be possible to apply the biotinylated effector before the internalization occurs. We only allowed 2 hr between the injection of avidin and bG4-Gd or bG4-Gd-SA in our experiments to avoid the internalization of biotinylated trastuzumab. However, the saturation of four avidin/streptavidin binding sites due to cross-linking of biotinylated antibody appeared to be the more dominant obstacle for the three-step pretargeting approach.

The failure of layer-by-layer multilayers composed of neutravidin-biotin-labeled antibody for sandwich fluorimmunosensing was recently reported (17). In that report the authors pointed out that virtually all the target-binding activity was derived from the final layer added and that additional layers provided no observable enhancement in fluorimmunoassay signal strength. While no explanation was provided, it can be easily attributed to the blocking of neutravidin binding sites due to cross-linking. Neutravidin binding sites may get saturated by the biotinylated antibody from the first layer and there would be no more biotin binding sites available for the next layer. The biotinavidin system is also widely used for signal amplification in immunohistochemistry. Different biotin-avidin schemes exist, such as BRAB (BRIdged Avidin Biotin) (18), ABC (Avidin Biotin Complex) (19), and LAB (Labeled Avidin Biotin) (18). The BRAB approach is identical to the three-step pretargeting approach that utilizes biotin-avidin. It is worth noting that only ABC and LAB are currently in common use (20). It is reasonable to argue that cross-linking of the biotinylated primary antibody and the partial to complete saturation of the avidin binding sites lead to the disappointing performance of BRAB.

The extended retention of bG4-Gd and bG4-Gd-SA in the body, especially in the kidneys, is of concern. The blood α half-life for G4D-(1B4M-Gd) and DTPA-Gd was 2.5 ± 0.9 min and 0.4 ± 0.2 min, and β half-life for G4D-(1B4M-Gd) and DTPA-Gd was 35 ± 7 min, and 51 ± 12 min, respectively, in mice (21). However, at 48 hr about 75% of DTPA-Gd is excreted, while only about 10% of G4D-(1B4M-Gd) is excreted, mainly in the urine (7). We expected

that our bG4-Gd and bG4-Gd-SA bears similar pharmacokinetics to that of G4D-(1B4M-Gd), as the main difference between them is the chelating agent. bG4-Gd, bG4-Gd-SA, and G4D-(1B4M-Gd) all showed strong kidney uptake, with the last being suggested for use as a kidney imaging agent (22). G4D has a measured diameter of 4.5 nm and G4D-(1B4M-Gd) was estimated to be about 6 nm in diameter (22). Kidney glomerular capillary walls are reported to have albumin restrictive small pores with radius in the range of 37–60 Å (23). G4D and its gadolinium complexes with an estimated radius under 30 Å should be able to pass the kidney glomerular barrier and be excreted to urine readily. However, this is not what we and others observed (6). It is possible that many of these kidney ultrafiltration studies were performed with dextran or Ficoll, which are not reabsorbed by the proximal tubules and the sieving coefficients were directly determined from the urine-to-plasma concentration ratios. As a result, kidney uptake of the macromolecules was not taken into account. More important, renal retention of macromolecules that contain chelated metal ions can be very different. It was well known that renal retention of radioactivity is a common and often dose-limiting side effect of radionuclide therapy with radiolabeled antibody fragments and peptides (24). Coadministration of positively charged lysine presented mixed results in reducing the kidney uptake of radionuclides (24,25). Surface charge did not appear to be a major factor in determining the pharmacokinetics of G4D-based gadolinium complexes since both bG4-Gd and bG4-Gd-SA showed extended kidney uptake. Molecular size is possibly the determining factor. Although the mechanism of kidney ultrafiltration is certainly beyond the scope of this article, it does appear that smaller-sized contrast agents will be excreted faster. Ultimately, the balance between the need for larger molecules for longer blood retention time and smaller molecules for faster excretion is a fine one. Biodegradable macromolecule contrast agents may provide an answer to this dilemma (26).

CONCLUSIONS

PAMAM dendrimer generation 4-based gadolinium agents bG4-Gd and bG4-Gd-SA were tested in BT-474 tumor-bearing mice, as well as MCF-7 and BT-474 bilateral tumor-bearing mice in a three-step pretargeting approach. These agents can accumulate in the tumor tissues through the EPR effect. A pretargeting three-step approach using the biotin-avidin/streptavidin recognition system produced no significant additional tumor enhancement, possibly due to the saturation of the avidin/streptavidin binding sites by biotinylated trastuzumab, and subsequent binding of bG4-Gd and bG4-Gd-SA was therefore blocked.

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FIG. 1. Fluorescence microscopy images of BT-474 cells labeled with biotinylated trastuzumab streptavidin-Alexa488 (left), and biotinylated G4D-Alexa594 (middle) sequentially. An overlay of both fluorescence images is shown on the right.

FIG. 2. Axial T_1 -weighted images (upper panels) and axial T_1 maps (lower panels) of mice treated with a three-step pretargeting approach that included biotinylated trastuzumab, avidin, and bG4D-Gd (middle). Precontrast images are shown on the left and images of a nontargeted three-step approach that included trastuzumab, avidin, and bG4D-Gd are shown on the right. Tumors are indicated by arrows.

FIG. 3.

Coronal T_1 -weighted images of mice treated with a three-step pretargeting approach that included biotinylated trastuzumab, avidin, and bG4D-Gd (middle). A precontrast T_1 image is shown at left and an image of a nontargeted three-step approach that included trastuzumab, avidin, and bG4D-Gd is shown at right. Tumors are indicated by arrows.

FIG. 4. Axial T_1 -weighted images (upper panels) and axial T_1 maps (lower panels) of mice treated with a three-step pretargeting approach that included biotinylated trastuzumab, avidin, and bG4D-Gd-SA (middle). Precontrast images are shown at left and images of a nontargeted three-step approach that included trastuzumab, avidin, and bG4D-Gd-SA are shown at right. Tumors are indicated by arrows.

FIG. 5.

Axial T_1 -weighted images of a mouse treated with 3 mg bG4D-Gd only. Images were taken at 15 min (left) and 24 hr (right) after the injection of bG4D-Gd. Tumors are indicated by arrows.

FIG. 6. Coronal (left) and axial T_1 -weighted (right) images taken 101 days after the injection of 6 mg bG4D-Gd-SA.

FIG. 7.

Graphic representation of a three-step pretargeting approach. Left: the presumed process includes (1) accumulation of the biotinylated antibody at the tumor site, (2) addition of avidin and its binding to the biotinylated antibody at the tumor, and (3) addition of biotinylated imaging agent and its binding to avidin-biotinylated antibody at the tumor. Right: in the real process all four avidin binding sites may be saturated by biotinylated antibody and the biotinavidin bridge is blocked. The biotinylated imaging agent loses its target and fails to reach the tumor.

Table 1

Summary of Measured BT-474 Tumor T_1 Values in Seconds

	bG4D-GD-SA		bG4D-Gd	
	3-Step Targeting	3-Step Nontargeting	3-Step Targeting	3-Step Nontargeting
Precontrast	1.9 ± 0.2	1.9 ± 0.3	2.3 ± 0.2	2.03 ± 0.09
24 hr postcontrast	1.6 ± 0.2	1.3 ± 0.2	1.4 ± 0.1	1.2 ± 0.1
				<i>bG4D-Gd Alone</i>
				1.9 ± 0.3
				1.4 ± 0.2

Table 2

Summary of Measured MCF-7 and BT-474 Bilateral Tumor T_1 Values in Seconds by Three-Step Pretargeting Approach

	MCF-7	BT-474
Precontrast	2.3 ± 0.2	2.3 ± 0.2
24 hr postcontrast	1.7 ± 0.2	1.8 ± 0.2