Published in final edited form as:

J Magn Reson Imaging. 2015 April ; 41(4): 1079–1087. doi:10.1002/jmri.24654.

Imaging of Brain Tumors With Paramagnetic Vesicles Targeted to Phosphatidylserine

Patrick M. Winter, PhD¹, John Pearce, BS¹, Zhengtao Chu, MD², Christopher M. McPherson, MD³, Ray Takigiku, PhD⁴, Jing-Huei Lee, PhD⁵, and Xiaoyang Qi, PhD^{2,*}

¹Department of Radiology, Cincinnati Children's Hospital, Cincinnati, Ohio, USA

²Department of Hematology and Oncology, University of Cincinnati, Cincinnati, Ohio, USA

³Division of Surgical Neuro-Oncology, University of Cincinnati, Cincinnati, Ohio, USA

⁴Bexion Pharmaceuticals LLC, Covington, Kentucky, USA

⁵Center for Imaging Research, University of Cincinnati, Cincinnati, Ohio, USA

Abstract

Purpose—To investigate paramagnetic saposin C and dioleoylphosphatidylserine (SapC-DOPS) vesicles as a targeted contrast agent for imaging phosphatidylserine (PS) expressed by glioblastoma multiforme (GBM) tumors.

Materials and Methods—Gd-DTPA-BSA/SapC-DOPS vesicles were formulated, and the vesicle diameter and relaxivity were measured. Targeting of Gd-DTPA-BSA/SapC-DOPS vesicles to tumor cells in vitro and in vivo was compared with nontargeted paramagnetic vesicles (lacking SapC). Mice with GBM brain tumors were imaged at 3, 10, 20, and 24 h postinjection to measure the relaxation rate (R1) in the tumor and the normal brain.

Results—The mean diameter of vesicles was 175 nm, and the relaxivity at 7 Tesla was 3.32 (s*mM)⁻¹ relative to the gadolinium concentration. Gd-DTPA-BSA/SapC-DOPS vesicles targeted cultured cancer cells, leading to an increased R1 and gadolinium level in the cells. In vivo, Gd-DTPA-BSA/SapC-DOPS vesicles produced a 9% increase in the R1 of GBM brain tumors in mice 10 h postinjection, but only minimal changes (1.2% increase) in the normal brain. Nontargeted paramagnetic vesicles yielded minimal change in the tumor R1 at 10 h postinjection (1.3%).

Conclusion—These experiments demonstrate that Gd-DTPA-BSA/SapC-DOPS vesicles can selectively target implanted brain tumors in vivo, providing noninvasive mapping of the cancer biomarker PS.

Keywords

cancer; MRI; contrast agent; glioblastoma multiforme

BRAIN TUMORS ARE associated with extremely poor prognosis in both adult and pediatric patients with a 5-year survival rate of only 30% (1). It is estimated that nearly 70,000 new cases of brain tumor were diagnosed in the US in 2013 (1). In particular, glioblastoma multiforme (GBM) is a highly aggressive and drug-resistant form of primary brain tumor (2–4). Despite the use of radiation and chemotherapy, the median survival for GBM patients is less than 15 months (5,6). Novel imaging methods that can help characterize brain tumors, aid in the selection of an effective therapy, and monitor the therapeutic response in these patients could greatly improve their survival rate and quality of life.

Vesicles formulated with saposin C and dioleoylphosphatidylserine (SapC-DOPS) have been shown to selectively target tumors, but not normal tissues (7–12). Formulating SapC-DOPS vesicles with an MRI visible beacon would allow noninvasive tracking of the uptake of this tumor specific agent. The high resolution, lack of ionizing radiation, excellent tissue penetration, and soft tissue contrast available with MRI provides distinct advantages over other molecular imaging approaches, such as nuclear or optical imaging. Traditional anatomical MRI and nonspecific contrast agents can localize a tumor mass and assess the abnormal vasculature feeding the tumor, but molecular imaging with a specifically targeted contrast agent could provide biochemical characterization of the disease, which could guide the selection of an effective treatment or allow early detection of the therapeutic effect.

Saposins are small nonenzymatic glycoproteins present in all normal tissues that act as biological activators of lysosomal enzymes (13). The functional organization of SapC consists of a membrane fusogenic domain and a region for activation of lysosomal enzymes (14,15). SapC associates with lipid membranes by embedding into membrane leaflets. Each helical peptide of SapC at the amino and carboxyl ends of the molecule is essential for insertion into lipid bilayers (16). SapC preferentially interacts with unsaturated, negatively charged phospholipids, such as phosphatidylserine (PS) (15,16). PS is present in all cells at relatively high amounts, approximately 2–10% of total cellular lipid (17). However, in normal cells, PS is only present on the intracellular aspect of the cellular membrane and not on the cell surface. In contradistinction, PS is relatively abundant on the surface of tumor cells and on tumor vasculature (18,19). Furthermore, the exposure level of PS on the cancer cell surface can be modulated by the metastatic potential (20) and at early stages of cell death (21,22), providing a metric of overall prognosis and therapeutic effect. Elevated PS levels have been observed in cultured brain tumor cells and tumors in vivo by flow cytometry using annexin A5 and immunohistochemical analysis with PS-specific binding proteins or anti-PS monoclonal antibodies (8,12). Specifically, GBM tumors express PS on the cell surface allowing fluorescent SapC-DOPS vesicles to selectively target these cells in vitro and in vivo (12). In vivo fluorescent imaging of SapC-DOPS vesicles has shown preferential tumor uptake and complete clearance from normal tissues by 24 h postinjection (9).

Tumor selective imaging of SapC-DOPS vesicles by means of MRI has been previously reported by encapsulating iron oxide particles inside the vesicles (9). In this study, we explored the use of a paramagnetic gadolinium chelate for noninvasively tracking SapC-DOPS vesicles. Gadolinium based MRI contrast agents are widely used clinically to detect

lesions and image blood vessels. At the present time, there are nine different gadolinium based contrast agents approved by the U.S. Food and Drug Administration (FDA) for clinical use (23). On the other hand, no iron oxide agents are currently commercially available for MRI (24). A superparamagnetic iron oxide nanoparticle agent called Ferumoxytol has been used for MRI (25,26), but it is only FDA approved for the treatment of anemia (27). In addition, gadolinium agents are often preferred because they generate an increase in the MRI signal, compared with iron oxide agents that typically generate a decrease in the MRI signal (28). The aim of this research was to investigate paramagnetic SapC-DOPS vesicles as a targeted contrast agent for imaging GBM cells in vitro and in vivo in the mouse brain.

MATERIALS AND METHODS

Preparation of Paramagnetic SapC-DOPS Vesicles

A lipophilic gadolinium chelate, Gadolinium-DTPA-bis(stearylamide) (Gd-DTPA-BSA, Avanti Polar Lipids, Inc., Alabaster, AL), was incorporated onto the surface of SapC-DOPS vesicles. The phospholipids, DOPS (Avanti Polar Lipids, Inc.), SapC (Changji Biotech Co., China), and Gd-DTPA-BSA, were combined at a molar ratio of 5:1:5 in phosphate buffered saline (PBS; Sigma-Aldrich Co., St. Louis, MO) and sonicated (Branson 1510 Ultrasonic Cleaner, Branson Ultrasonics, Danbury, CT) for 20 min at 4°C. The vesicle diameter and polydispersity was measured by dynamic light scattering (N4 plus particle size analyzer, Coulter, Miami, FL) (9,14). Transmission electron microscopy (TEM) was performed with a JEOL JEM1230 microscope (JEOL USA, Inc. Peabody, MA) using an AMT Advantage Plus digital camera (Advanced Microscopy Techniques, Corp., Woburn, MA). Vesicle samples were stored at room temperature or 4°C for 13 days and particle sizing was repeated to assess the stability of the formulation. The gadolinium content of the vesicle solution was measured by inductively coupled plasma spectrometry (ICP, Agilent Technologies, Tokyo, Japan).

Paramagnetic vesicles were imaged at 7 Tesla (T) (Bruker, Billerica, MA) to measure the relaxivity compared with a standard MRI contrast agent, Gd-DTPA. Samples of paramagnetic Gd-DTPA-BSA/SapC-DOPS vesicles were serially diluted in normal saline (Sigma-Aldrich Co., St. Louis, MO) to gadolinium concentrations of 0.88, 0.35, and 0.11 mM, while the Gd-DTPA sample was diluted to 0.56 mM Gd. A saline sample was also imaged to measure the relaxation rate in the absence of gadolinium. The gadolinium content of all the vesicle and Gd-DTPA samples was measured by ICP. The longitudinal relaxation rate (R1) of each sample was measured with variable repetition time (TR) rapid acquisition with refocused echoes (RARE) pulse sequences using a 72 mm quadrature birdcage coil (Bruker, Billerica, MA). To accurately measure higher R1s (larger than 1.3 1/s), nine TR values ranging from 100 to 3000 ms were collected, leading to a total scan time of 4 min and 22 s. For the lower R1s, 13 TR values ranging from 100 to 12,000 ms were collected, leading to a total scan time of 22 min and 16 s. For both measurements, a single 1.5-mm-thick slice was imaged, with 1 signal average, echo time (TE) of 24.7 ms, RARE factor of 4, 90° excitation pulse, 180° refocusing pulse, 128 × 128 matrix and 400 × 400 µm in-plane

resolution. The R1 of each sample was determined by means of pixel-by-pixel fitting of the MRI signal intensity versus the TR using the equation:

$$S_{TR} = A \times (1 - e^{-TR \times R1})$$

where S_{TR} is the signal intensity at a given TR, A is the maximum amplitude of the MRI signal, and TR and R1 are defined above. All image fitting and analysis routines were written in Matlab R2012a (The Mathworks, Inc., Natick, MA). The R1 value was plotted against the gadolinium concentration of each sample and fit to a straight line to determine the relaxivity.

Cell Culture Studies

The in vitro targeting ability of our MRI contrast agent was studied by adding Gd-DTPA-BSA/SapC-DOPS vesicles to the media of cultured human GBM cells (Gli36, a gift from Dr. Balveen Kaur at the Ohio State University, Columbus, OH). Previous studies with Gli36 cells have shown that they express PS on the cell surface allowing fluorescent SapC-DOPS vesicles to target the cells in vitro (12). Cells were cultured in 6 well plates (BD Biosciences, San Jose, CA) with Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich Co., St. Louis, MO) to a density of approximately 1.26×10^6 cells per well. Targeted Gd-DTPA-BSA/SapC-DOPS vesicles and nontargeted control vesicles (lacking SapC) were formulated as described above and used within 24 h for the cell culture experiments. The cells were treated for 2 h with either no vesicles, nontargeted vesicles ($[Gd] = 880 \mu M$), or Gd-DTPA-BSA/SapC-DOPS vesicles diluted to 14 or 55 μM Gd. After incubation, the cells were washed, trypsinized off the plates, fixed in 2% paraformaldehyde (Sigma-Aldrich Co., St. Louis, MO) and centrifuged into pellets. Cell pellets were imaged at 7T using a 72-mm quadrature birdcage coil. An R1 map was calculated from a variable TR RARE pulse sequence with a total scan time of 11 min and 11 s. The 11 TR values ranged from 175 to 6000 ms, TE was 25.2 ms, 3 slices were imaged, 1 mm slice thickness, 0.5 mm slice gap, 1 signal average, RARE factor of 4, 90° excitation pulse, 180° refocusing pulse, 256×128 matrix and $195 \times 390 \mu m$ in-plane resolution. The gadolinium content of each cell pellet was measured by ICP.

In Vivo Studies

The in vivo targeting ability of Gd-DTPA-BSA/SapC-DOPS vesicles was tested in mice with intracranial brain tumors (Human GBM U87 EGFR-Luc cells, a gift from Dr. Balveen Kaur at the Ohio State University, Columbus, OH). U87 tumors express PS on the cell surface and have been imaged in vivo with fluorescent SapC-DOPS vesicles (12). All animal procedures were approved by the Institutional Animal Care and Use Committee of Cincinnati Children's Hospital Medical Center. Human GBM cells (10^5 cells in 4 μL of PBS) were injected into the right hemisphere of 4-week-old nude female mice (NCr-Nu/Nu, National Cancer Institute, Frederick, MD) under stereotactic guidance ($n = 17$). A separate group of animals ($n = 5$) had saline injected into the right hemisphere of the brain to serve as sham controls. MRI was performed 10 days after tumor or sham injection at 7T to measure the R1 relaxation rate and T1-weighted image intensity of the tumor and contralateral brain.

Targeted Gd-DTPA-BSA/SapC-DOPS vesicles and non-targeted control vesicles (lacking SapC) were formulated as described above and used within 24 h for the in vivo experiments. GBM (n = 10) and sham (n = 5) animals were imaged before and 3, 10, 20, and 24 h after intravenous injection of 200 μ L of Gd-DTPA-BSA/SapC-DOPS vesicles containing 0.88 mM Gd-DTPA-BSA, 2 μ g/ μ L SapC and 0.906 μ g/ μ L DOPS. To investigate the nonspecific accumulation of vesicles within the brain tumors, GBM mice (n = 7) were imaged before and 3 and 10 h after intravenous injection of 200 μ L of nontargeted paramagnetic vesicles.

The U87 tumor cells injected into the mice are genetically engineered to express luciferase. Following the MRI experiments, animals were injected with potassium luciferin (Goldbio, St. Louis, MO) and ex vivo bioluminescence imaging was used to confirm GBM growth with an IVIS 200 Series imaging system with an XFO-6 fluorescent kit (Xenogen, Alameda, CA) (8–10,12). For histology, the brains were fixed in formalin, embedded in paraffin, and cut into 5 μ m sections. Tissue sections were stained with hematoxylin–eosin as described previously (7,10,12). The histology slides were reviewed by Dr. Winter and Dr. Qi to verify tumor growth. Drs. Winter and Qi have over 20 years of combined experience reviewing histology sections of animal cancer models.

In Vivo MRI Protocol

Mice were anesthetized with isoflurane (2% in 2 L/min oxygen) and placed in a custom-built single turn solenoid coil with a diameter of 2.2 cm and a length of 3 cm. A reference phantom filled with deionized water spiked with 0.5 mM Gd-DTPA was placed next to the animal for normalization of the MRI signal at different time points. The animal body temperature was monitored with a fiber optic temperature probe and maintained at 37°C with a computer controlled warm air blower (SA Instruments, Inc., Stony Brook, NY). The tumor was localized with an axial two-dimensional (2D) T2-weighted RARE sequence with a scan time of 3 min and 44 s. The TR was 3500 ms, TE was 44 ms, 11 slices were imaged, 0.75 mm slice thickness, 0.25 mm slice gap, four signal averages, RARE factor of 16, 90° excitation pulse, 180° refocusing pulse, 256 $\times$ 256 matrix and 130 $\times$ 130 μ m in-plane resolution. Then the brain was imaged with a 2D T1-weighted sequence with identical resolution and geometry (TR was 250 ms, TE was 10.38 ms, four signal averages, scan time of 4 min and 16 s). A variable TR sequence was collected to map the R1 values (11 TR values ranging from 150 to 8000 ms, TE of 23.2 ms, 1 axial slice, 1 mm slice thickness, 1 signal average, RARE factor of 4, 90° excitation pulse, 180° refocusing pulse, 256 $\times$ 128 matrix, 200 $\times$ 100 μ m in-plane resolution, scan time of 13 min and 45 s). The R1 mapping slice was positioned on the burr hole of the skull to consistently cover the same area of tumor and brain at each imaging time point. Regions of interest encompassing the tumor, the normal brain or the reference phantom were manually drawn on the T2-weighted images and mapped onto the corresponding R1 map or T1-weighted image slices. The T1-weighted signal intensity was normalized relative to the reference phantom for calculating changes in the image intensity at different time points.

Statistical Analysis

All experimental results are reported as mean $\pm$ standard error where appropriate. Statistical differences between group means was evaluated using analysis of variance in SAS (Cary, NC) with a P value of 0.05 as the threshold for statistical significance.

RESULTS

Incorporation of Gd-DTPA-BSA into SapC vesicles resulted in a particle size of 175 nm and a polydispersity of 0.119. TEM imaging of Gd-DTPA-BSA/SapC-DOPS vesicles magnified 50,000 times revealed spherical, unilamellar structures similar to those (Fig. 1) observed in previous preparations developed for imaging or therapeutic applications (8–10). The paramagnetic formulation was very stable over 13 days of storage at both 4°C and room temperature. Storage at 4°C caused the particle size to increase by 1.0% per day and the polydispersity to increase by 1.5% per day. Storage at room temperature resulted in a 1.8% increase in particle size per day and an 8.5% increase in polydispersity per day. The gadolinium content of the Gd-DTPA-BSA/SapC-DOPS vesicles was 0.88 mM as measured by ICP. At 7T, Gd-DTPA had a relaxivity of $2.80 \text{ (s}^*\text{mM)}^{-1}$ relative to the gadolinium concentration (Fig. 2). As expected for a paramagnetic contrast agent, the R1 of Gd-DTPA-BSA/SapC-DOPS vesicles increased linearly with the gadolinium concentration. Fitting these data to a straight line yielded a relaxivity of $3.32 \text{ (s}^*\text{mM)}^{-1}$ with a correlation coefficient of 0.99. Because each vesicle carried approximately 2000 gadolinium chelates, the relaxivity per particle was $6640 \text{ (s}^*\text{mM)}^{-1}$.

Gd-DTPA-BSA/SapC-DOPS vesicles successfully targeted cultured human GBM cells (Fig. 3) increasing the relaxation rate of the cells in a dose dependent manner. The R1 of untreated cells was $0.384 \pm 0.004 \text{ s}^{-1}$. Cells treated with a high concentration (880 μM Gd) of untargeted paramagnetic vesicles (lacking SapC) had a R1 of $0.490 \pm 0.006 \text{ s}^{-1}$ ($P < 0.05$ versus cells alone). With targeting, a similar R1 value ($0.475 \pm 0.016 \text{ ms}$, $P < 0.05$ versus cells alone) was obtained with a 64-fold lower concentration of vesicles (14 μM Gd). Increasing the concentration of targeted paramagnetic vesicles to 55 μM Gd further increased the R1 to $0.600 \pm 0.020 \text{ ms}$ ($P < 0.05$ versus cells alone, nontargeted cells and cells treated with 14 μM Gd). While toxicology was not explicitly tested, incubation with Gd-DTPA-BSA/SapC-DOPS or nontargeted vesicles did not induce any noticeable cell death in the GBM cultures. ICP measurements of the gadolinium content of the cell pellets agreed with the MRI results. The untreated cells showed no gadolinium. Treatment with nontargeted paramagnetic vesicles produced a similar amount of gadolinium as treatment with 64-fold lower concentration of targeted paramagnetic vesicles, 7.8 ± 2.4 versus $6.0 \pm 1.7 \text{ }\mu\text{M}$ Gd, respectively. Incubating the cells with a higher concentration of targeted paramagnetic vesicles (55 μM Gd), yielded $63 \pm 2 \text{ }\mu\text{M}$ Gd in the pellet ($P < 0.05$ versus cells alone, nontargeted cells and cells treated with 14 μM Gd).

Ten days after injection of GBM cells, the intracranial tumors reached an average size of $18.3 \pm 4.7 \text{ mm}^3$. Before injection of the Gd-DTPA-BSA/SapC-DOPS vesicles, the R1 of the tumor, contralateral normal brain, and sham injected brain was $0.467 \pm 0.010 \text{ s}^{-1}$, $0.559 \pm 0.003 \text{ s}^{-1}$ and $0.521 \pm 0.034 \text{ s}^{-1}$, respectively. The tumor R1 was significantly lower than the R1 of the normal brain ($P < 0.05$), but there was no difference between the normal brain

and the sham injected brain ($P>0.05$). In vivo mapping of the tumor R1 after injection of Gd-DTPA-BSA/SapC-DOPS vesicles showed a marked increase at 3 and 10 h, followed by a return to the baseline value by 24 h postinjection (Fig. 4). The R1 increased relatively uniformly in the tumor, indicating that PS is uniformly expressed in the tumor and that the vesicles could penetrate into the tumor mass. The average change in the tumor R1 value was $9.0 \pm 2.3\%$ at 10 h postinjection, while the normal brain and the sham injected brain showed no clear increase, $1.2 \pm 1.5\%$ and $1.4 \pm 1.9\%$, $P<0.05$ versus tumor R1 (Fig. 5). Similarly, injection with non-targeted paramagnetic vesicles (lacking SapC) showed no change in the tumor R1 at 10 h postinjection, $1.3 \pm 1.3\%$, $P<0.05$ versus tumor R1 with Gd-DTPA-BSA/SapC-DOPS vesicles (Fig. 5). The tumor R1 remained elevated ($7.9 \pm 1.5\%$, $P<0.05$) compared with the normal and sham brain at 20 h postinjection, but was statistically identical to the controls at 24 h postinjection ($4.7 \pm 2.0\%$, $P>0.05$).

The increased R1 relaxation rate observed in the tumor following injection of Gd-DTPA-BSA/SapC-DOPS vesicles would be expected to produce signal enhancement on T1-weighted MRI. The T1-weighted images were normalized relative to a reference positioned within the field of view, allowing comparison of the MRI signal intensity at different time points. The tumor region displayed a relatively uniform enhancement at 3 and 10 h postinjection (Fig. 6). As with the R1 mapping results, the contrast agent enhancement did not appear to be constrained to the tumor periphery. At 10 h postinjection, the T1-weighted signal intensity increased by $3.7 \pm 2.6\%$ in the tumor, while the signal in the normal brain was essentially unchanged, $-0.5 \pm 1.2\%$, $P<0.05$ (Fig. 7). This difference persisted up to 20 h postinjection (tumor: $4.1 \pm 2.8\%$, normal brain: $-1.6 \pm 1.1\%$, $P<0.05$). In agreement with the R1 mapping results, the T1-weighted signal returned to the baseline values by 24 h postinjection of Gd-DTPA-BSA/SapC-DOPS vesicles.

Following the MRI experiments, the brains of tumor-bearing mice and sham mice were removed for ex vivo bioluminescence imaging (Fig. 8). High bioluminescence signal was observed at the injection site of the tumor mice, confirming GBM tumor growth. In the sham injected animals, no bioluminescence signal was observed at the injection site, although some background signal was present in other areas of the brain. Histology of the brain tumors showed a predominance of viable tumor cells without significant necrosis or hemorrhage (Fig. 9).

DISCUSSION

Incorporating Gd-DTPA-BSA into the phospholipid membrane of SapC-DOPS vesicles yielded a novel MRI contrast agent targeted to PS. Gd-DTPA-BSA/SapC-DOPS vesicles had high relaxivity and stability. The vesicles selectively targeted cultured cancer cells and GBM tumors implanted in mice, while nontargeted paramagnetic vesicles displayed much lower tumor uptake.

This study demonstrates that SapC-DOPS vesicles can be formulated with a paramagnetic chelate on the surface to generate T1-weighted contrast in MRI. The applications of SapC-DOPS vesicles for fluorescent imaging and cancer therapy have been previously reported (9,10,12). Furthermore, MRI tracking of SapC-DOPS vesicles with iron oxide particles and

T2*-weighted MRI has been recently reported (9). However, this required a multi-step formulation method consisting of coupling iron oxide particles to the DOPS lipid, formulating the vesicles, dissociating the iron oxide particles on the outer surface of the vesicles and separating free iron particles from the SapC-DOPS vesicles. In contrast, incorporating paramagnetic gadolinium onto the vesicles only requires addition of a lipophilic chelate to the other components before vesicle formulation. Moreover, paramagnetic contrast agents are routinely used in clinical MRI, while iron oxide particles are not currently FDA approved as MRI contrast agents (24).

After injection of Gd-DTPA-BSA/SapCDOPS vesicles, the tumor R1 increased uniformly throughout the tumor area. This result suggests that PS is uniformly expressed throughout the GBM tumors and that the vesicles are able to penetrate into the tumor mass. Several cancer biomarkers have been reported to be predominately localized to the tumor rim, such as angiogenic markers (29–32) and fibrin (33). Previous studies using fluorescence imaging have demonstrated that SapC-DOPS vesicles generate uniform enhancement within a tumor (9,10,12). The iron oxide-coupled SapC-DOPS vesicles also displayed a uniform increase in MRI relaxation across the tumor (9), although subcutaneously implanted tumors were studied rather than the intracranial tumors reported here. The ability of MRI molecular imaging to localize signal enhancement to individual regions of the tumor demonstrate a notable advantage over other imaging modalities, such as optical and nuclear imaging, which typically do not have sufficient spatial resolution to visualize sub-regions of a tumor.

MRI, however, does not have the spatial resolution to determine if Gd-DTPA-BSA/SapC-DOPS vesicles are targeting the tumor cells directly, or if the agent is binding to the tumor vasculature, which also expresses high levels of PS (18). A previous study tracked the microscopic cellular uptake of fluorescent SapC-DOPS vesicles (12). Mice with tumors that expressed green fluorescent protein were injected with SapC-DOPS vesicles and the tumors were removed for histology. Fluorescence microscopy of frozen tissue sections showed that the fluorescent signal from SapC-DOPS vesicles colocalized with the green fluorescent protein signal from the tumor cells and not with other cell types. That study suggests that Gd-DTPA-BSA/SapC-DOPS vesicles would predominately target the tumor cells themselves, rather than the tumor vasculature.

The present results showed that the T1-weighted MRI signal enhancement registered in GBM tumors was considerably lower than the change in R1. This result arose from the suboptimal imaging parameters in our T1-weighted imaging sequence. To achieve the maximum contrast between the pre- and postinjection R1 values, a T1-weighted sequence with a TR around 2000 ms could have been used, or a smaller flip angle to achieve similar contrast with a shorter TR. The T1-weighted sequence we used had a TR of 250 ms, which would be expected to produce 3–4 times less contrast. Further refinements and optimization of the pulse sequences, the contrast agent formulation, the postinjection imaging time point, the tumor size and the contrast agent dose would be expected to increase the MRI signal enhancement observed in this study.

The majority of PS imaging studies reported in the literature has used nuclear imaging tracers (34–36), often consisting of annexin A5 as the targeting ligand. Positron emission

tomography (PET) and single-photon emission computed tomography (SPECT) can track the uptake of annexin A5 that has been chemically modified with a radioactive group. With this approach, clinical PET and SPECT imaging of annexin A5 in patients with breast, head and neck, and lung cancer have shown that higher PS signal following treatment correlates with a better outcome (37–39). These nuclear imaging studies clearly demonstrate potential clinical uses of PS targeted contrast agents. There have been other MRI agents, however, that have been developed for molecular imaging of PS expression. Some of these agents were only validated in cell culture preparations. For example, a bimodal MRI and optical imaging agent was produced by encapsulating quantum dots within a micelle that incorporated paramagnetic chelates on the outer surface (40). Human recombinant annexin A5 proteins were covalently coupled to the micelles for targeting. The ability of these bimodal annexin A5-conjugated micelles to target PS was demonstrated in cultured apoptotic cells by both fluorescence imaging and MRI. In another in vitro study, the utility of a gadolinium-containing liposome agent was compared with an iron-containing agent for MRI of apoptotic cells (41). Gadolinium increased the R1 of the cells producing a positive signal enhancement on T1-weighted images, while iron increased the R2 of the cells and decreased the signal on T2-weighted images. The two agents also had very different diameters, with the paramagnetic liposome being 100 nm in diameter and the iron oxide based micelle having a 10 nm diameter. Cells treated with these agents showed a 258% increase in the R1 value (paramagnetic liposomes) and a 172% increase in the R2 (iron oxide micelles). As an alternative to annexin A5, the C2A domain of synaptotagmin has also been shown to bind PS and image apoptotic cells when coupled to an iron oxide label (42). In addition, the C2A domain has been coupled to gadolinium chelates through biotin-avidin binding and used to image apoptosis in a subcutaneous tumor model in vivo (43). This agent was cleared through the kidneys with a blood half-life around 200 min. Mice bearing subcutaneous tumors were treated with etoposide and cyclophosphamide to induce apoptosis, and serial MRI revealed retention of the targeted agent up to 24 h postinjection. In treated tumors, the targeted contrast agent increased the R1 by 19%. In two control groups, treated animals receiving a nontargeted contrast agent and untreated animals receiving the targeted contrast agent, minimal changes in the R1 were observed, 2% and 0%, respectively. In our experiments, the Gd-DTPA-BSA/SapC-DOPS vesicles were used to target an orthotopic brain tumor model, further illustrating the clinical potential of a targeted MRI contrast agent that can detect cancer biomarkers in vivo.

While these studies show that Gd-DTPA-BSA/SapC-DOPS vesicles can selectively target tumors in vivo and generate sufficient MRI signal, there are a couple of important limitations to this research. First, the animals in these experiments had rather large tumors that could be easily localized with T2-weighted MRI. Further studies are warranted to define the minimum tumor size that can be detected with these paramagnetic vesicles. Both the sensitivity of MRI to the paramagnetic contrast agent as well as the PS expression levels of tumors with different sizes would be expected to strongly impact the ability to detect uptake of the vesicles as the tumor grows. Furthermore, while these studies clearly demonstrate that Gd-DTPA-BSA/SapC-DOPS vesicles induce changes in the MRI signal of GBM tumors, it is not clear that the MRI signal is directly proportional to the expression level of PS. Additional experiments will be needed to correlate MRI enhancement with PS expression

levels and to measure the sensitivity of the MRI signal to changes in PS expression, such as during chemotherapy.

In conclusion, these experiments demonstrate that Gd-DTPA-BSA/SapC-DOPS vesicles can target brain tumors in vivo, providing noninvasive mapping of the cancer biomarker PS. A tumor-targeted MRI contrast agent could provide invaluable information during diagnosis, treatment planning or long term monitoring of patients suffering from brain tumors or other cancers.

Acknowledgments

Contract grant sponsor: Mayfield Education and Research Foundation; Contract grant sponsor: New Drug State Key Project; Contract grant number: 009ZX09102-205; Contract grant sponsor: NIH/NCI; Contract grant number: 1R01CA158372-01; Contract grant sponsor: Division of Hematology-Oncology at University of Cincinnati; Contract grant sponsor: the Cincinnati Children's Hospital Research Foundation.

X.Q., C.M.M., and R.T. were supported in part by Mayfield Education and Research Foundation, and X.Q. was funded by New Drug State Key Project and the NIH/NCI.

References

1. Dolecek TA, Propp JM, Stroup NE, Kruchko C. CBTRUS statistical report: primary brain and central nervous system tumors diagnosed in the United States in 2005–2009. *Neuro Oncol.* 2012; 14(Suppl 5):v1–v49. [PubMed: 23095881]
2. de Vries NA, Beijnen JH, Boogerd W, van Tellingen O. Blood-brain barrier and chemotherapeutic treatment of brain tumors. *Expert Rev Neurother.* 2006; 6:1199–1209. [PubMed: 16893347]
3. Pardridge WM. The blood-brain barrier: bottleneck in brain drug development. *NeuroRx.* 2005; 2:3–14. [PubMed: 15717053]
4. Westphal M, Lamszus K. The neurobiology of gliomas: from cell biology to the development of therapeutic approaches. *Nat Rev Neurosci.* 2011; 12:495–508. [PubMed: 21811295]
5. Stupp R, Hegi ME, Mason WP, et al. Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial. *Lancet Oncol.* 2009; 10:459–466. [PubMed: 19269895]
6. Wen PY, Kesari S. Malignant gliomas in adults. *N Engl J Med.* 2008; 359:492–507. [PubMed: 18669428]
7. Abu-Baker S, Chu Z, Stevens AM, Li J, Qi X. Cytotoxicity and Selectivity in Skin Cancer by SapC-DOPS Nanovesicles. *J Cancer Ther.* 2012; 3:321–326. [PubMed: 25485166]
8. Chu Z, Abu-Baker S, Palascak MB, Ahmad SA, Franco RS, Qi X. Targeting and Cytotoxicity of SapC-DOPS Nanovesicles in Pancreatic Cancer. *PLoS One.* 2013; 8:e75507. [PubMed: 24124494]
9. Kaimal V, Chu Z, Mahller YY, et al. Saposin C coupled lipid nano-vesicles enable cancer-selective optical and magnetic resonance imaging. *Mol Imaging Biol.* 2011; 13:886–897. [PubMed: 20838909]
10. Qi X, Chu Z, Mahller YY, Stringer KF, Witte DP, Cripe TP. Cancer-selective targeting and cytotoxicity by liposomal-coupled lysosomal saposin C protein. *Clin Cancer Res.* 2009; 15:5840–5851. [PubMed: 19737950]
11. Unruh D, Turner K, Srinivasan R, et al. Alternatively spliced tissue factor contributes to tumor spread and activation of coagulation in pancreatic ductal adenocarcinoma. *Int J Cancer.* 2014; 134:9–20. [PubMed: 23754313]
12. Wojton J, Chu Z, Mathsyaraja H, et al. Systemic delivery of SapC-DOPS has antiangiogenic and antitumor effects against glioblastoma. *Mol Ther.* 2013; 21:1517–1525. [PubMed: 23732993]
13. Darmon A, Maschmeyer P, Winau F. The immunological functions of saposins. *Adv Immunol.* 2010; 105:25–62. [PubMed: 20510729]

14. Qi X, Chu Z. Fusogenic domain and lysines in saposin C. *Arch Biochem Biophys*. 2004; 424:210–218. [PubMed: 15047193]
15. Qi X, Qin W, Sun Y, Kondoh K, Grabowski GA. Functional organization of saposin C. Definition of the neurotrophic and acid beta-glucosidase activation regions. *J Biol Chem*. 1996; 271:6874–6880. [PubMed: 8636113]
16. Qi X, Grabowski GA. Differential membrane interactions of saposins A and C: implications for the functional specificity. *J Biol Chem*. 2001; 276:27010–27017. [PubMed: 11356836]
17. Vance JE, Steenbergen R. Metabolism and functions of phosphatidylserine. *Prog Lipid Res*. 2005; 44:207–234. [PubMed: 15979148]
18. Ran S, Thorpe PE. Phosphatidylserine is a marker of tumor vasculature and a potential target for cancer imaging and therapy. *Int J Radiat Oncol Biol Phys*. 2002; 54:1479–1484. [PubMed: 12459374]
19. Utsugi T, Schroit AJ, Connor J, Bucana CD, Fidler IJ. Elevated expression of phosphatidylserine in the outer membrane leaflet of human tumor cells and recognition by activated human blood monocytes. *Cancer Res*. 1991; 51:3062–3066. [PubMed: 2032247]
20. Riedl S, Rinner B, Asslaber M, et al. In search of a novel target -phosphatidylserine exposed by non-apoptotic tumor cells and metastases of malignancies with poor treatment efficacy. *Biochim biophys Acta*. 2011; 1808:2638–2645. [PubMed: 21810406]
21. Balasubramanian K, Schroit AJ. Aminophospholipid asymmetry: a matter of life and death. *Annu Rev Physiol*. 2003; 65:701–734. [PubMed: 12471163]
22. Zhao D, Stafford JH, Zhou H, Thorpe PE. Near-infrared optical imaging of exposed phosphatidylserine in a mouse glioma model. *Transl Oncol*. 2011; 4:355–364. [PubMed: 22191000]
23. Yang L, Krefting I, Gorovets A, et al. Nephrogenic systemic fibrosis and class labeling of gadolinium-based contrast agents by the Food and Drug Administration. *Radiology*. 2012; 265:248–253. [PubMed: 22923714]
24. Deddens LH, Van Tilborg GA, Mulder WJ, De Vries HE, Dijkhuizen RM. Imaging neuroinflammation after stroke: current status of cellular and molecular MRI strategies. *Cerebrovasc Dis*. 2012; 33:392–402. [PubMed: 22456323]
25. Hasan DM, Amans M, Tihan T, et al. Ferumoxytol-enhanced MRI to image inflammation within human brain arteriovenous malformations: a pilot investigation. *Translational stroke research*. 2012; 3(Suppl 1):166–173. [PubMed: 23002401]
26. McCullough BJ, Kolokythas O, Maki JH, Green DE. Ferumoxytol in clinical practice: implications for MRI. *J Magn Reson Imaging*. 2013; 37:1476–1479. [PubMed: 23097302]
27. Schwenk MH. Ferumoxytol: a new intravenous iron preparation for the treatment of iron deficiency anemia in patients with chronic kidney disease. *Pharmacotherapy*. 2010; 30:70–79. [PubMed: 20030475]
28. Pauleit D, Textor J, Bachmann R, et al. Hepatocellular carcinoma: detection with gadolinium- and ferumoxides-enhanced MR imaging of the liver. *Radiology*. 2002; 222:73–80. [PubMed: 11756708]
29. Boles KS, Schmieder AH, Koch AW, et al. MR angiogenesis imaging with Robo4- vs. alphaVbeta3-targeted nanoparticles in a B16/F10 mouse melanoma model. *FASEB J*. 2010; 24:4262–4270. [PubMed: 20585027]
30. Jarzyna PA, Deddens LH, Kann BH, et al. Tumor angiogenesis phenotyping by nanoparticle-facilitated magnetic resonance and near-infrared fluorescence molecular imaging. *Neoplasia*. 2012; 14:964–973. [PubMed: 23097630]
31. Winter PM, Caruthers SD, Kassner A, et al. Molecular imaging of angiogenesis in nascent Vx-2 rabbit tumors using a novel alpha(-nu)beta3-targeted nanoparticle and 1.5 tesla magnetic resonance imaging. *Cancer Res*. 2003; 63:5838–5843. [PubMed: 14522907]
32. Winter PM, Schmieder AH, Caruthers SD, et al. Minute dosages of alpha(nu)beta3-targeted fumagillin nanoparticles impair Vx-2 tumor angiogenesis and development in rabbits. *Faseb J*. 2008; 22:2758–2767. [PubMed: 18362202]
33. Uppal R, Medarova Z, Farrar CT, Dai G, Moore A, Caravan P. Molecular imaging of fibrin in a breast cancer xenograft mouse model. *Invest Radiol*. 2012; 47:553–558. [PubMed: 22960948]

34. Blankenberg FG, Strauss HW. Nuclear medicine applications in molecular imaging. *J Magn Reson Imaging*. 2002; 16:352–361. [PubMed: 12353251]
35. Lahorte CM, Vanderheyden JL, Steinmetz N, Van de Wiele C, Dierckx RA, Slegers G. Apoptosis-detecting radioligands: current state of the art and future perspectives. *Eur J Nucl Med Mol Imaging*. 2004; 31:887–919. [PubMed: 15138718]
36. Yang TJ, Haimovitz-Friedman A, Verheij M. Anticancer therapy and apoptosis imaging. *Exp Oncol*. 2012; 34:269–276. [PubMed: 23070012]
37. Belhocine T, Steinmetz N, Hustinx R, et al. Increased uptake of the apoptosis-imaging agent (99m)Tc recombinant human Annexin V in human tumors after one course of chemotherapy as a predictor of tumor response and patient prognosis. *Clin Cancer Res*. 2002; 8:2766–2774. [PubMed: 12231515]
38. Kartachova MS, Valdes Olmos RA, et al. 99mTc-HYNIC-rh-annexin-V scintigraphy: visual and quantitative evaluation of early treatment-induced apoptosis to predict treatment outcome. *Nucl Med Commun*. 2008; 29:39–44. [PubMed: 18049096]
39. Rottey S, Slegers G, Van Belle S, Goethals I, Van de Wiele C. Sequential 99mTc-hydrazinonicotinamide-annexin V imaging for predicting response to chemotherapy. *J Nucl Med*. 2006; 47:1813–1818. [PubMed: 17079815]
40. van Tilborg GA, Mulder WJ, Chin PT, et al. Annexin A5-conjugated quantum dots with a paramagnetic lipidic coating for the multimodal detection of apoptotic cells. *Bioconjug Chem*. 2006; 17:865–868. [PubMed: 16848390]
41. van Tilborg GA, Mulder WJ, Deckers N, et al. Annexin A5-functionalized bimodal lipid-based contrast agents for the detection of apoptosis. *Bioconjug Chem*. 2006; 17:741–749. [PubMed: 16704213]
42. Schellenberger EA, Sosnovik D, Weissleder R, Josephson L. Magneto/optical annexin V, a multimodal protein. *Bioconjug Chem*. 2004; 15:1062–1067. [PubMed: 15366960]
43. Krishnan AS, Neves AA, de Backer MM, et al. Detection of cell death in tumors by using MR imaging and a gadolinium-based targeted contrast agent. *Radiology*. 2008; 246:854–862. [PubMed: 18187402]

Figure 1. Typical TEM micrograph of a Gd-DTPA-BSA/ SapC-DOPS vesicle magnified 50,000 times. The vesicles were spherical and unilamellar with a diameter of ~175 nm, similar to previous preparations.

Figure 2.

Relaxivity of Gd-DTPA-BSA/SapC-DOPS vesicles at 7T compared with Gd-DTPA. The vesicles and Gd-DTPA displayed similar relaxivities relative to the gadolinium content, 3.32 and 2.80 (s*mM)⁻¹, respectively. However, because each vesicle incorporated approximately 2000 gadolinium chelates, the relaxivity per particle was 6640 (s*mM)⁻¹.

Figure 3.

R1 maps (7T) of cell pellets after treatment with Gd-DTPA-BSA/SapC-DOPS vesicles demonstrate specific targeting of GBM cells. Treating cultured cells with increasing amounts of Gd-DTPA-BSA/SapC-DOPS vesicles (0, 14, and 55 μM Gd) produced progressive increases in the R1 and the gadolinium concentration measured by ICP.

Figure 4.

Tumor R1 (s^{-1}) before and after injection of Gd-DTPA-BSA/SapC-DOPS vesicles. A T2-weighted image (T2-w Image) is shown in the upper right panel to provide clear definition of the tumor within the brain (arrow). The colorcoded R1 values progressively increase at 3 and 10 h postinjection. At 24 h postinjection, the R1 map appears to have a similar value as the preinjection image.

Figure 5.

Percent change in R1 after injection of Gd-DTPA-BSA/SapC-DOPS vesicles in the tumor (“Tumor”), contralateral normal brain (“Brain”), and sham brain (“Sham”), and R1 change in the tumor after injection of nontargeted vesicles (lacking SapC, “NT Vesicles”). At 10 and 20 h postinjection, the increase in R1 was significantly higher in the tumor (9.0% and 7.9%) compared with the normal brain (1.2% and –1.0%) or sham brain (1.4% and –0.3%), $*P < 0.05$. The nontargeted vesicles only generated minimal R1 changes in the tumor at 3 and 10 h postinjection (0.7% and 1.3%). By 24 h postinjection, the R1 values were similar in the tumor, normal brain and sham brain ($P > 0.05$).

Figure 6.

A: T2-weighted MRI of tumor implanted in the brain of a mouse. **B:** The T1-weighted MRI intensity of the tumor region was pseudo-colored from blue to red (normalized to the reference placed under the animal's head) and overlaid onto the grayscale T1-weighted image. The T1-weighted signal increased from baseline to 3 h and 10 h postinjection of Gd-DTPA-BSA/SapC-DOPS vesicles. At 24 h postinjection, the T1-weighted signal returned to the baseline value.

Figure 7.

T1-weighted signal enhancement for the tumor (“Tumor”) and contralateral normal brain (“Brain”) postinjection of Gd-DTPA-BSA/SapC-DOPS vesicles. At 10 and 20 h postinjection, the signal enhancement was significantly higher in the tumor (3.7% and 4.1%) compared with the normal brain (−0.5% and −1.6%, $*P < 0.05$). By 24 h postinjection, the T1-weighted signal was similar in both tissues ($P > 0.05$).

Figure 8.

Ex vivo bioluminescence imaging confirmed tumor growth in the brain injected with U87 EGFR-Luc cells (right) and not in the brain injected with saline (left). In the tumor brain, signal is observed at the injection site (red arrow). In the sham treated brain, some background signal is seen in other areas of the brain, but no signal is observed at the injection site.

Figure 9.

A representative histologic tissue section from an orthotopic xenografted brain tumor stained with hematoxylin–eosin. **Top:** The low magnification image shows a highly localized tumor mass with sharp borders and no infiltration into areas of normal brain tissue. **Bottom:** The higher magnification images show a dense collection of viable tumor cells without significant necrosis or hemorrhage.