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Imaging the delivery of brain-penetrating PLGA nanoparticles in the brain using magnetic resonance

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Abstract

Current therapy for glioblastoma multiforme (GBM) is largely ineffective, with nearly universal tumor recurrence. The failure of current therapy is primarily due to the lack of approaches for the efficient delivery of therapeutics to diffuse tumors in the brain. In our prior study, we developed brain-penetrating nanoparticles that are capable of penetrating brain tissue and distribute over clinically relevant volumes when administered via convection-enhanced delivery (CED). We

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Conflict of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

demonstrated that these particles are capable of efficient delivery of chemotherapeutics to diffuse tumors in the brain, indicating that they may serve as a groundbreaking approach for the treatment of GBM. In the original study, nanoparticles in the brain were imaged using positron emission tomography (PET). However, clinical translation of this delivery platform can be enabled by engineering a non-invasive detection modality using magnetic resonance imaging (MRI). For this purpose, in this study, we developed chemistry to incorporate superparamagnetic iron oxide (SPIO) into the brain-penetrating nanoparticles. We demonstrated that SPIO-loaded nanoparticles, which remain the same morphology as nanoparticles without SPIO, have an excellent transverse (T_2) relaxivity. After CED, the distribution of nanoparticles in the brain (i.e., in the vicinity of injection site) can be detected using MRI and the long-lasting signal attenuation of SPIO-loaded brain-penetrating nanoparticles lasted over a one-month timecourse. Development of these nanoparticles is significant as, in future clinical applications, co-administration of SPIO-loaded nanoparticles will allow for intraoperative monitoring of particle distribution in the brain to ensure drug-loaded nanoparticles reach tumors as well for monitoring the therapeutic benefit with time and to evaluate tumor relapse patterns.

Keywords

brain-penetrating nanoparticles; SPIO; MRI; malignant gliomas; PLGA

Introduction

Glioblastoma is most common malignant tumor of the CNS, with an incidence of approximately 18,500 cases per year [1]. Despite decades of research, glioblastoma remains one of the most deadly cancers known: overall survival has remained static since the advent of multimodal surgical/radiation/temozolomide-based care, with long-term survivors comprising only 10% of all patients [2–4]. Tremendous progress has been made to improve our understanding of glioblastoma biology by studying genomics [5–12], cellular hierarchy [13–19], microenvironment [20–22], and vasculature [23], but none of these advances has yet been translated into verifiable survival benefit.

The difficulty in producing effective therapies is thought to be secondary to two factors: poor drug delivery and inadequate tumor sensitivity. An ideal therapy for glioblastoma, therefore, must be highly penetrative—to reach cells that have migrated into regions far from the site of tumor initiation—and must carry a payload that is effective against the cell subpopulation most responsible for tumor initiation, growth, vascularization, and therapeutic resistance. To address drug delivery challenges, we have developed brain-penetrating nanoparticles, which are in size smaller than 70 nm and in non-aggregating form, a delivery vehicle comprised of the FDA-approved copolymer poly(*lactic-co-glycolic acid*) (PLGA) [24]. It was previously reported that the brain interstitial spaces in normal brain are between 38 and 64 nm [25] while are 70–100 nm in regions with tumor [26]. Therefore, nanoparticles in 70nm may accumulate in brain tumors more efficient than in normal brain tissue. We found that these nanoparticles were able to disperse over a clinically relevant volume in the brain when administered via convection-enhanced delivery (CED), which disperses agents via both convection and diffusion [27–30]. When loaded with drugs with *in vitro* efficacy

against brain cancer stem cells (BCSCs), brain-penetrating nanoparticles produce unprecedented survival in a BCSC-derived xenograft model of glioblastoma. We also developed methods for conjugation of *N*-(4-[¹⁸F]fluorobenzyl)propanamido ([¹⁸F]NPB4) to the surface of nanoparticles and demonstrated that, with this modification, delivery of brain-penetrating nanoparticles in the brain can be non-invasively monitored using positron emission tomography (PET) [24].

Non-invasive monitoring of the delivery of brain-penetrating nanoparticles in the brain is a significant development. First, it makes possible the intraoperative monitoring of particle distribution from the injection site in the brain to ensure drug-loaded nanoparticles reach dispersed tumor cells. Second, it makes it possible to monitor the therapeutic benefit with time and to evaluate tumor relapse patterns with respect to the known pattern of drug delivery. While PET/CT is extremely useful in diagnostic, and other contexts, it is costly—and the short half-life of PET isotopes[31]—makes it difficult to use for monitoring of drug delivery to and in the brain.

Magnetic resonance imaging (MRI) is an attractive alternative approach, as MRI is routinely used clinically to visualize brain tumors before and after surgery. Therefore, functionalization of nanoparticles to allow visualization by MRI will facilitate clinical translation of brain-penetrating nanoparticles. One potential approach is to conjugate gadolinium contrast agents to the surface of nanoparticles. However, this approach has limitations for engineering nanoparticles for drug delivery to the brain: gadolinium-based contrast agents require water access for MRI detection, so they typically need to be displayed on the surface of nanoparticles to generate sufficient signal [32]. Unfortunately, surface modification alters particle size and surface charge, potentially changing the penetrative ability of nanoparticles in brain tissue. Alternatively, superparamagnetic iron oxide (SPIO) can be used. SPIO is an excellent T2 agent that has been experimentally demonstrated to function as a contrast agent for both therapeutic and diagnostic purposes in the mouse brain [33], rat brain [34], as well as in large brain of dogs [35]. Several forms of SPIO have been approved by the FDA for use in a variety of clinical settings [36–39]. Unlike gadolinium contrast agents, MRI contrast due to SPIO does not depend on water access, so that SPIO can be encapsulated inside of nanoparticles without altering the physical properties of brain-penetrating nanoparticles [39]. Therefore, SPIO can be an excellent contrasts for encapsulation into brain-penetrating nanoparticles. In this study, we synthesized SPIO-loaded brain-penetrating nanoparticles, demonstrated their T2 relaxivities and detection at low concentration by MRI after CED to the brain, and demonstrated that these nanoparticles have minimal toxicity on normal brain tissue.

Methods

Chemicals

PLGA 50:50 with an inherent viscosity of 0.59 dL/g was purchased from Lactel Polymers, Inc. (Pelham, AL, USA)) and used as received. All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture

Human glial cell line SVG p12 was purchased from ATCC (American Type Culture Collection) and were grown at 37°C incubator containing 5% CO₂ and cultured in MEM medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 100 units/mL penicillin and 100 µg/mL streptomycin (Invitrogen). Human neural stem cell line ReNcell CX were purchased from Millipore and were maintained in NSC Maintenance Media (Millipore) supplemented with Basic fibroblast growth factor (bFGF) and Epidermal growth factor (EGF).

Synthesis of SPIO

SPIO was synthesized as previously reported[39]. Briefly, iron acetylacetonate (Fe(acac)₃) (0.396 g, 1.56 mmol), oleic acid (1.47 mL, 4.64 mmol), oleylamine (1.02 mL, 3.09 mmol), 1,2-hexadecanediol (2.005 g, 7.76 mmol) and benzyl ether (10 mL) were added to a single-neck round bottom flask equipped with a magnetic stir bar and a condenser and deoxygenated for an hour. The reaction was gradually heated to 200 °C and held at that temperature for 3 hours and then allowed to cool room temperature. The reaction mixture was precipitated in ethanol and centrifuged twice. Ethanol was decanted and the product was dried via nitrogen purge leaving a black powder.

Synthesis and characterization of SPIO-loaded brain-penetrating nanoparticles

Nanoparticles were synthesized according to our recently published procedures with minor modifications [24]. Briefly, 100 mg of PLGA and 20 mg SPIO were dissolved in 2 mL of chloroform. The polymer/drug solution was then added dropwise to 4 mL of 2.5% polyvinyl alcohol (PVA) as the outer aqueous phase and sonicated to form an emulsion. The emulsion was poured into a beaker containing aqueous 0.3% (v/v) PVA and stirred at room temperature for 3 h to allow the solvent to evaporate and particles to harden. Then, the nanoparticle solution was first centrifuged at low speed (8,000 × g for 10 min) to pellet the large particles. The supernatant was decanted and small nanoparticles were collected through high-speed ultracentrifugation (100,000 × g for 30 min, × 2). To prevent nanoparticle aggregation during lyophilization, trehalose was added to the final aqueous solution at a ratio of 0.5:1 (trehalose:nanoparticles) by mass immediately prior to lyophilization. The size of nanoparticles and loading of SPIO in nanoparticles were determined using the same procedures described in our recent reports [24, 39].

Cell proliferation assay

The standard MTT assay was used to quantify the effects of nanoparticles on cell proliferation. Briefly, cells were cultured in 96-well plates (Falcon). Twenty-four hours later, cells were treated with nanoparticles in a range of 0.05–1 mg/ml. Three days after treatment, medium was removed and replaced with fresh medium containing 10% MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma) solution (4.14 mg/mL). Four hours after incubation at 37°C, all media was removed. Formazan was dissolved in DMSO and the optical density (O.D.) was measured at 590 nm. The relative inhibition on growth was determined using the following formula: Growth inhibition = (control O.D. – sample O.D.)/control O.D.

In vitro MR relaxivity measurements

Four NMR tubes containing samples of different concentrations of SPIO-loaded nanoparticles (0, 0.2, 0.4 and 0.6 mg/ml) were used for relaxivity measurements. The longitudinal (R_1) and transverse (R_2) relaxation rate maps were obtained at 9.4T using an Agilent horizontal-bore spectrometer (Agilent Technologies Inc., Santa Clara, CA, USA) with a ^1H radio frequency (RF) volume coil of 4 cm diameter and at 4.0T using a Bruker horizontal-bore spectrometer (Bruker, Billerica, MA, USA) with a ^1H RF volume coil of 4 cm diameter. A spin-echo pulse sequence was used at both 9.4T and 4.0T to image a single 128×128 slice of 2 mm thickness with a field of view (FOV) of $28 \times 28 \text{ mm}^2$. For R_1 mapping, 11 different repetition time (TR) values in the range of 50 to 8000 ms were used, with an echo-time (TE) of 9ms at 9.4T and 15ms at 4.0T. For R_2 mapping at 9.4T, 12 different TE values in the range from 9 to 500 ms were used, with the same TR of 6000ms. For R_2 mapping at 4.0T, 11 different TE values in the range from 15 to 500 ms were used, with the same TR of 6000ms. The R_1 and R_2 values were calculated by fitting the time dependence of the absolute MR intensity to a single exponential function. The r_1 and r_2 relaxivities at the two magnetic fields were calculated from the slope of the linear fit of R_1 or R_2 versus SPIO concentration, $[\text{SPIO}]$, according to the equation:

$$R_i = R_i^0 + r_i [\text{SPIO}] \quad [1]$$

where R_i^0 ($i=1$ or 2) represents water R_1 or R_2 , respectively, in the absence of the SPIO-loaded nanoparticles.

CED of SPIO-Loaded brain-penetrating nanoparticles

All animal experimental procedures on rats were approved by the Yale Institutional Animal Care and Use Committee (IACUC). CED was performed as previously described [24]. Sprague-Dawley rats were first anesthetized with ketamine/xylazine. Animals were then prepped with betadine and alcohol and placed in a stereotactic frame. A linear midline incision was made and a burr hole was drilled in the skull 3mm lateral and 0.5 mm anterior to bregma. A 26G Hamilton syringe with 28G stepdown inner cannula, was inserted to a depth of 5mm. The tissue was allowed to equilibrate mechanically for 5 minutes. SPIO-loaded nanoparticles in PBS were infused continuously at a rate of $0.667 \mu\text{L}/\text{min}$, which was selected based on our previous studies [24, 40]. Following infusion, the syringe was left in place for 5 min, after which it was removed. The burr hole was filled with bone wax (Lukens, Reading PA), the scalp was closed with surgical staples, and the rat was removed to a clean cage with free access to food and water mixed with ibuprofen for analgesia.

In vivo MR Image Acquisition and Analysis

Immediately after initial CED of SPIO-containing brain-penetrating nanoparticles, the animals ($n=4$) were artificially ventilated (70% N_2O , 30% O_2) and kept under 1.5% isoflurane anesthesia. The spin-echo MR datasets were obtained using the 4.0T Bruker horizontal-bore spectrometer (Bruker, Billerica, MA, USA) with a ^1H RF surface coil of 2.5 cm diameter, positioned on top of the animal head. 16 coronal slices of 128×128 resolution and 1 mm thickness were acquired using a FOV of $32 \times 32 \text{ mm}^2$, a TR of 6s and 8 different TE values in the range from 15 to 115 ms, resulting in an in-plane resolution of 250×250

μm^2 . The T_2 maps were obtained in Matlab (MathWorks, Inc., Natick, MA, USA) by fitting the absolute MR intensity versus TE in each voxel to a single exponential function. Measurement of nanoparticle volume, V_d , was obtained from both spin-echo images and T_2 maps using BioImage Suite (<http://www.bioimagesuite.org/>) as follows. First the original spin-echo images and the T_2 maps were resampled to 200 μm isotropic resolution. Next, each spin-echo image intensity was normalized to its maximum value to provide similar intensity threshold for each dataset. Finally, the 3D volume occupied by the SPIO-loaded nanoparticles, V_d , was calculated at each TE using four intensity threshold values (15, 20, 25 and 30%). In addition, V_d was also estimated from the T_2 maps. In this case, the volume was given by the regions of the brain with $T_2 < 80\text{ms}$. The lifetime τ of the SPIO-loaded nanoparticles in the rat brain, was estimated from the exponential decrease of V_d/V_i over time (t) according to:

$$V_d/V_i = (V_d/V_i)_0 e^{-t/\tau} \quad [2]$$

where $(V_d/V_i)_0$ represents the ratio between the volume V_d immediately after the CED of SPIO-containing brain-penetrating nanoparticles (day 0) and the injected volume V_i .

Results

Characterization of SPIO-Loaded brain-penetrating nanoparticles

SEM of SPIO-loaded brain-penetrating nanoparticles showed that nanoparticles were approximately 68 ± 6 nm diameter and morphologically spherical (Fig. 1A and 1B), consistent with previously published reports [24]. To characterize these nanoparticles in terms of MR relaxivity properties, we conducted R_1 and R_2 relaxation rate measurements of water signal at various nanoparticle concentrations, at two different magnetic field strengths, 9.4T and 4.0T. The r_1 and r_2 relaxivities were calculated from the slope of the linear fit of R_1 (Fig. 1C) and R_2 (Fig. 1D) versus nanoparticle concentration according to eq.1 (Table 1). The r_1 values are small at both field strengths, $0.003 \pm 0.002 \text{ s}^{-1}\text{mg}^{-1}\text{ml}$ at 9.4T and $0.006 \pm 0.005 \text{ s}^{-1}\text{mg}^{-1}\text{ml}$ at 4.0T (Fig. 1C), indicating that the SPIO-loaded nanoparticles are very weak longitudinal (T_1) relaxation agents. In contrast, the r_2 relaxivities are high, $236 \pm 10 \text{ s}^{-1}\text{mg}^{-1}\text{ml}$ at 9.4T and $202 \pm 21 \text{ s}^{-1}\text{mg}^{-1}\text{ml}$ at 4.0T (Fig. 1D). These r_2 relaxivities are comparable to previously published values[39] and suggest these SPIO-loaded brain-penetrating nanoparticles as excellent transverse (T_2) relaxation agents. We previously found that what enables SPIO-loaded PLGA nanoparticles to be superparamagnetic agent is the heavy loading of paramagnetic iron oxide per nanocarrier, as nanoparticles without SPIO did not produce MRI signal [39]. Therefore, in the rest of this study, we did not evaluate nanoparticles without SPIO encapsulation.

SPIO-Loaded Brain-Penetrating Nanoparticles Distribute Over a Large Volume

We sought to assess the dose-dependency of the volume occupied by the SPIO-loaded brain-penetrating nanoparticles delivered using CED. Immediately after CED of 20 μl of fluid containing SPIO-loaded nanoparticles, spin-echo MR images at various TE were acquired at 4.0T at three different nanoparticle doses, 0.02 mg, 0.1 mg and 0.5 mg, injected in three different animals (Fig. 2A–C). Using these images, T_2 maps were also generated (Fig. 2D–

F). The hypointensity observed in the right caudate is due to the presence of the SPIO-loaded brain-penetrating nanoparticles. The measured V_d depends on the dose injected, TE and the intensity threshold value used (Supplementary Figure 1). V_d increases with the nanoparticle dose at the same TE and threshold value. At the same TE, V_d increases with increased threshold value, for all three doses investigated. However, V_d increases with TE (at the same threshold value) only at 0.02 mg and 0.1 mg doses, while at 0.5 mg the volume remained almost unchanged with increasing TE. V_d was also measured using the T_2 maps (Fig. 2D–F). In this case, V_d was defined as the volume for which $T_2 < 80$ ms and the calculated values were 50 μ l at 0.02 mg, 71 μ l at 0.1 mg and 76 μ l at 0.5 mg, which are similar to V_d values calculated using the spin-echo images at higher echo times, TE > 80 ms (Supplementary Figure 1), where the MR contrast is maximized by longer TE. Examples of 3D distributions of the SPIO-loaded nanoparticles at 4.0T are shown in Supplementary Fig. 2 for the three doses investigated, at 30% intensity threshold and a TE of 72 ms.

The nanoparticle volume of distribution, V_d , and V_d/V_i (where $V_i = 20$ μ l represents the injected nanoparticle volume) are properties determined by size and the electrochemical surface of the brain-penetrating nanoparticles [24]. Using V_d estimation from the T_2 maps, the calculated V_d/V_i values were 2.5, 3.6 and 3.8 for a nanoparticle dose of 0.02 mg, 0.1 mg and 0.5 mg, respectively. The V_d/V_i values obtained using spin-echo MR images at TE of 72 ms and 30% intensity threshold were 2.3, 3.7 and 4.6 for a nanoparticle dose of 0.02 mg, 0.1 mg and 0.5 mg, respectively.

SPIO-Loaded Brain-Penetrating Nanoparticles Produce Long-Lasting MR Signal Attenuation

Two different doses of SPIO-loaded brain-penetrating nanoparticles, 0.5 mg in 20 μ l and 0.25 mg in 10 μ l, were infused intracranially using CED in two different rats for each dose and evaluated over a four-week period using MR imaging. Examples of MR images obtained from a rat infused with 0.5 mg in 20 μ l are shown in Fig. 3. For this animal, using the time dependence of V_d/V_i given by eq. 2, we calculated a lifetime $\tau = 89 \pm 9$ days (Supplementary Figure 3, circles). Using the V_d/V_i values from all 4 animals investigated, we calculated an average V_d/V_i value at different times (3, 7, 14, 21 and 28 days) during the four-week period (Supplementary Figure 3). The average V_d/V_i value immediately after the infusion (day 0) was 3.41 ± 0.15 . Interestingly, the V_d/V_i values from all 4 animals investigated were very similar at all time-points (less than 10% standard deviation for each time-point) and were independent of injected volume V_i (Supplementary Figure 3). A lifetime $\tau = 65 \pm 11$ days was calculated from the non-linear fit of the time-dependence of the average V_d/V_i values according to eq. 2, shown with a continuous line in Supplementary Figure 3.

SPIO-Loaded Brain-Penetrating Nanoparticles Are Non-Toxic

At the end of longitudinal imaging experiments, the rats which received a total nanoparticle dose of 0.5 mg in 20 μ l were sacrificed and the brain was harvested for blinded histologic analysis (Fig. 4). Tissue is notably unremarkable at low and medium magnifications. Focusing on white matter tracts at highest magnification, yellow-stained macrophages are noted, indicating likely cellular uptake of SPIO or SPIO-loaded particles. We further tested

SPIO-loaded brain-penetrating nanoparticles on normal human glial cell line SVG p12 and normal human neural stem cell line ReNcell CX and found they had limited toxicity on both cells (Supplementary Figure 4). This is an expected finding and has previously been described [34].

Discussion

The combination of the brain-penetrating nanoparticle delivery system and a BCSC-targeted small molecule agent has previously been shown to increase survival in a BCSC-derived rat xenograft model of glioblastoma [24]. In this previous work, the nanoparticles were surface modified with a positron-emitting isotope, allowing visualization of the nanoparticles by PET imaging: the infused brain-penetrating nanoparticles (~70 nm diameter) distribute over a volume 8-fold larger than slightly larger nanoparticles (~150 nm diameter) of the same composition. However, clinical translation and, ultimately, clinical utility will be aided by lowering the cost, radiation, and transience of imaging modality. Therefore, functionalizing nanoparticles for MRI is important for translating these nanoparticles for future clinical applications.

In this study, we developed methods to synthesize SPIO-loaded brain-penetrating nanoparticles. We demonstrated that these nanoparticles have an excellent transverse relaxivity r_2 , which is higher than $200 \text{ s}^{-1}\text{mg}^{-1}\text{ml}$ at both 4.0T and 9.4T (Table 1) and consistent with previously measured values [39]. We performed the rest of the study at 4.0T, which represents the closest clinical field strength available, to ensure that the results from this study are clinically relevant and translatable. We found that administration of SPIO-loaded nanoparticles at a concentration of 5 mg/ml (0.1 mg in 20 μl) was sufficient to monitor their distribution in the brain, as the V_d estimations by MR imaging at this concentration aligned with those determined using microscopy or PET signal [24]. We also found that the strong MRI signal from the SPIO-loaded nanoparticles lasted over a long period, suggesting that a single administration of PLGA brain-penetrating nanoparticles allows controlled release of cargo agents over long time periods. The T_2 contrast derived from SPIO-loaded brain-penetrating nanoparticles is not dependent on SPIO release from brain-penetrating nanoparticles, as free SPIO released from brain-penetrating nanoparticles is cleared rapidly enough to exert no significant effect on T_2 weighted MR images [41]. Therefore, SPIO contained within brain-penetrating nanoparticles is the sole source of MR contrast in this system. As SPIO-loaded nanoparticles have minimal toxicity and have the same physical properties, including the morphology, size and surface property, as nanoparticles without SPIO, we speculate that a small amount (~5 mg/ml) of these particles can be co-administered with a relatively larger amount (up to 300 mg/ml) of drug-loaded nanoparticles to enable monitoring the delivery using MRI in future clinical application.

Numerous SPIO formulations have been developed and evaluated in the laboratory, with at least three having been tested in humans as diagnostic aides. The most commonly described formulations – ferumoxides (*Feridex*; Berlex Laboratories, Wayne, NJ, USA) [36], ferumoxytol (Advanced Magnetix, Inc., Lexington, MA, USA) [37], and ferumoxtran-10 (*Combidex*; Advanced Magnetix, Inc., Lexington, MA, USA) [38] – have been tested domestically and abroad as intravascular contrast agents in wide-ranging applications

including stroke [42], inflammatory and autoimmune disease [43], and, most recently, malignancy [44]. Combidex and Feridex are two dextran-coated SPIO formulations tested in humans. Different from Combidex – ferumoxtran-10, which is a completely coated iron oxide particle with diameter between 20 and 50nm, Feridex – ferumoxide – is an incompletely dextran-coated SPIO particle with diameter 60–185nm. In contrast, Ferumoxytol is an ultrasmall (~30nm) SPIO formulation incompletely coated with semisynthetic carbohydrate designed to remain inside the intravascular space [37]. Our preparations of SPIO-loaded brain-penetrating nanoparticles are unique in their functionality, as we demonstrate in this report. This long experience with the safety of SPIO in humans, coupled with the even longer history of use of PLGA in FDA-approved products, suggests that our preparations will also be safe and non-toxic.

In summary, we have developed techniques to synthesize and deliver PLGA brain-penetrating nanoparticles and monitor their distribution in the brain using MRI. In future clinical applications, non-invasive monitoring of the delivery of brain-penetrating nanoparticles in the brain can be potentially achieved through co-administration of independently synthesized SPIO- and drug-containing brain-penetrating nanoparticle populations that are otherwise identical. Because of the excellent safety profiles of PLGA and SPIO, this novel drug delivery platform can have an immediate impact on monitoring of treatment in patients with GBM.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Mrugala MM, Chamberlain MC. Mechanisms of disease: temozolomide and glioblastoma--look to the future. *Nature clinical practice Oncology*. 2008; 5:476–486.10.1038/ncponc1155
2. Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ, Belanger K, Brandes AA, Marosi C, Bogdahn U, Curschmann J, Janzer RC, Ludwin SK, Gorlia T, Allgeier A, Lacombe D, Cairncross JG, Eisenhauer E, Mirimanoff RO. European Organisation for R, Treatment of Cancer Brain T, Radiotherapy G, National Cancer Institute of Canada Clinical Trials G . Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *The New England journal of medicine*. 2005; 352:987–996.10.1056/NEJMoa043330 [PubMed: 15758009]
3. Stupp R, Hegi ME, Mason WP, van den Bent MJ, Taphoorn MJ, Janzer RC, Ludwin SK, Allgeier A, Fisher B, Belanger K, Hau P, Brandes AA, Gijtenbeek J, Marosi C, Vecht CJ, Mokhtari K, Wesseling P, Villa S, Eisenhauer E, Gorlia T, Weller M, Lacombe D, Cairncross JG, Mirimanoff RO. European Organisation for R, Treatment of Cancer Brain T, Radiation Oncology G, National Cancer Institute of Canada Clinical Trials G. 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. *The lancet oncology*. 2009; 10:459–466.10.1016/S1470-2045(09)70025-7 [PubMed: 19269895]

4. Wick W, Weller M, van den Bent M, Stupp R. Bevacizumab and recurrent malignant gliomas: a European perspective. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*. 2010; 28:e188–189. author reply e190-182. 10.1200/JCO.2009.26.9027 [PubMed: 20159801]
5. Parsons DW, Jones S, Zhang X, Lin JC, Leary RJ, Angenendt P, Mankoo P, Carter H, Siu IM, Gallia GL, Olivi A, McLendon R, Rasheed BA, Keir S, Nikolskaya T, Nikolsky Y, Busam DA, Tekleab H, Diaz LA Jr, Hartigan J, Smith DR, Strausberg RL, Marie SK, Shinjo SM, Yan H, Riggins GJ, Bigner DD, Karchin R, Papadopoulos N, Parmigiani G, Vogelstein B, Velculescu VE, Kinzler KW. An integrated genomic analysis of human glioblastoma multiforme. *Science*. 2008; 321:1807–1812.10.1126/science.1164382 [PubMed: 18772396]
6. Balss J, Meyer J, Mueller W, Korshunov A, Hartmann C, von Deimling A. Analysis of the IDH1 codon 132 mutation in brain tumors. *Acta neuropathologica*. 2008; 116:597–602.10.1007/s00401-008-0455-2 [PubMed: 18985363]
7. Benita Y, Kikuchi H, Smith AD, Zhang MQ, Chung DC, Xavier RJ. An integrative genomics approach identifies Hypoxia Inducible Factor-1 (HIF-1)-target genes that form the core response to hypoxia. *Nucleic acids research*. 2009; 37:4587–4602.10.1093/nar/gkp425 [PubMed: 19491311]
8. Verhaak RG, Hoadley KA, Purdom E, Wang V, Qi Y, Wilkerson MD, Miller CR, Ding L, Golub T, Mesirov JP, Alexe G, Lawrence M, O'Kelly M, Tamayo P, Weir BA, Gabriel S, Winckler W, Gupta S, Jakkula L, Feiler HS, Hodgson JG, James CD, Sarkaria JN, Brennan C, Kahn A, Spellman PT, Wilson RK, Speed TP, Gray JW, Meyerson M, Getz G, Perou CM, Hayes DN. Cancer Genome Atlas Research N. Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. *Cancer cell*. 2010; 17:98–110.10.1016/j.ccr.2009.12.020 [PubMed: 20129251]
9. Rao SK, Edwards J, Joshi AD, Siu IM, Riggins GJ. A survey of glioblastoma genomic amplifications and deletions. *Journal of neuro-oncology*. 2010; 96:169–179.10.1007/s11060-009-9959-4 [PubMed: 19609742]
10. Cerami E, Demir E, Schultz N, Taylor BS, Sander C. Automated network analysis identifies core pathways in glioblastoma. *PloS one*. 2010; 5:e8918.10.1371/journal.pone.0008918 [PubMed: 20169195]
11. Gerlinger M, Rowan AJ, Horswell S, Larkin J, Endesfelder D, Gronroos E, Martinez P, Matthews N, Stewart A, Tarpey P, Varela I, Phillimore B, Begum S, McDonald NQ, Butler A, Jones D, Raine K, Latimer C, Santos CR, Nohadani M, Eklund AC, Spencer-Dene B, Clark G, Pickering L, Stamp G, Gore M, Szallasi Z, Downward J, Futreal PA, Swanton C. Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. *The New England journal of medicine*. 2012; 366:883–892.10.1056/NEJMoa1113205 [PubMed: 22397650]
12. Sottoriva A, Spiteri I, Piccirillo SG, Touloumis A, Collins VP, Marioni JC, Curtis C, Watts C, Tavare S. Intratumor heterogeneity in human glioblastoma reflects cancer evolutionary dynamics. *Proceedings of the National Academy of Sciences of the United States of America*. 2013; 110:4009–4014.10.1073/pnas.1219747110 [PubMed: 23412337]
13. Singh SK, Clarke ID, Hide T, Dirks PB. Cancer stem cells in nervous system tumors. *Oncogene*. 2004; 23:7267–7273.10.1038/sj.onc.1207946 [PubMed: 15378086]
14. Clarke MF. Neurobiology: at the root of brain cancer. *Nature*. 2004; 432:281–282.10.1038/432281a [PubMed: 15549078]
15. Ohgaki H, Kleihues P. Epidemiology and etiology of gliomas. *Acta Neuropathol*. 2005; 109:93–108.10.1007/s00401-005-0991-y [PubMed: 15685439]
16. Jordan CT, Guzman ML, Noble M. Cancer stem cells. *The New England journal of medicine*. 2006; 355:1253–1261.10.1056/NEJMra061808 [PubMed: 16990388]
17. Keith B, Simon MC. Hypoxia-inducible factors, stem cells, and cancer. *Cell*. 2007; 129:465–472.10.1016/j.cell.2007.04.019 [PubMed: 17482542]
18. Fan X, Salford LG, Widegren B. Glioma stem cells: evidence and limitation. *Seminars in cancer biology*. 2007; 17:214–218.10.1016/j.semcancer.2006.04.002 [PubMed: 16750389]
19. Stiles CD, Rowitch DH. Glioma stem cells: a midterm exam. *Neuron*. 2008; 58:832–846.10.1016/j.neuron.2008.05.031 [PubMed: 18579075]

20. Soeda A, Park M, Lee D, Mintz A, Androutsellis-Theotokis A, McKay RD, Engh J, Iwama T, Kunisada T, Kassam AB, Pollack IF, Park DM. Hypoxia promotes expansion of the CD133-positive glioma stem cells through activation of HIF-1alpha. *Oncogene*. 2009; 28:3949–3959.10.1038/onc.2009.252 [PubMed: 19718046]
21. Seidel S, Garvalov BK, Wirta V, von Stechow L, Schanzer A, Meletis K, Wolter M, Sommerlad D, Henze AT, Nister M, Reifenberger G, Lundeberg J, Frisen J, Acker T. A hypoxic niche regulates glioblastoma stem cells through hypoxia inducible factor 2 alpha. *Brain : a journal of neurology*. 2010; 133:983–995.10.1093/brain/awq042 [PubMed: 20375133]
22. Charles NA, Holland EC, Gilbertson R, Glass R, Kettenmann H. The brain tumor microenvironment. *Glia*. 2011; 59:1169–1180.10.1002/glia.21136 [PubMed: 21446047]
23. Soda Y, Marumoto T, Friedmann-Morvinski D, Soda M, Liu F, Michiue H, Pastorino S, Yang M, Hoffman RM, Kesari S, Verma IM. Transdifferentiation of glioblastoma cells into vascular endothelial cells. *Proceedings of the National Academy of Sciences of the United States of America*. 2011; 108:4274–4280.10.1073/pnas.1016030108 [PubMed: 21262804]
24. Zhou J, Patel TR, Sirianni RW, Strohbehn G, Zheng M-Q, Duong N, Schafbauer T, Huttner AJ, Huang Y, Carson RE, Zhang Y, Sullivan DJ Jr, Piepmeyer JM, Saltzman WM. Highly penetrative, drug-loaded nanocarriers improve treatment of glioblastoma. *Proc Natl Acad Sci U S A*. 2013; 110:11751–11756. [PubMed: 23818631]
25. Thorne RG, Nicholson C. In vivo diffusion analysis with quantum dots and dextrans predicts the width of brain extracellular space. *Proc Natl Acad Sci U S A*. 2006; 103:5567–5572.10.1073/pnas.0509425103 [PubMed: 16567637]
26. Hobbs SK, Monsky WL, Yuan F, Roberts WG, Griffith L, Torchilin VP, Jain RK. Regulation of transport pathways in tumor vessels: role of tumor type and microenvironment. *Proc Natl Acad Sci U S A*. 1998; 95:4607–4612. [PubMed: 9539785]
27. Bobo RH, Laske DW, Akbasak A, Morrison PF, Dedrick RL, Oldfield EH. Convection-enhanced delivery of macromolecules in the brain. *Proceedings of the National Academy of Sciences of the United States of America*. 1994; 91:2076–2080. [PubMed: 8134351]
28. Sawyer AJ, Piepmeyer JM, Saltzman WM. New methods for direct delivery of chemotherapy for treating brain tumors. *The Yale journal of biology and medicine*. 2006; 79:141–152. [PubMed: 17940624]
29. Kunwar S, Chang S, Westphal M, Vogelbaum M, Sampson J, Barnett G, Shaffrey M, Ram Z, Piepmeyer J, Prados M, Croteau D, Pedain C, Leland P, Husain SR, Joshi BH, Puri RK, Group PS. Phase III randomized trial of CED of IL13-PE38QQR vs Gliadel wafers for recurrent glioblastoma. *Neuro-oncology*. 2010; 12:871–881.10.1093/neuonc/nop054 [PubMed: 20511192]
30. Sampson JH, Archer G, Pedain C, Wembacher-Schroder E, Westphal M, Kunwar S, Vogelbaum MA, Coan A, Herndon JE, Raghavan R, Brady ML, Reardon DA, Friedman AH, Friedman HS, Rodriguez-Ponce MI, Chang SM, Mittermeyer S, Croteau D, Puri RK, Investigators PT. Poor drug distribution as a possible explanation for the results of the PRECISE trial. *Journal of neurosurgery*. 2010; 113:301–309.10.3171/2009.11.JNS091052 [PubMed: 20020841]
31. Rahmim A, Zaidi H. PET versus SPECT: strengths, limitations and challenges. *Nuclear medicine communications*. 2008; 29:193–207.10.1097/MNM.0b013e3282f3a515 [PubMed: 18349789]
32. Ratzinger G, Agrawal P, Korner W, Lonkai J, Sanders HM, Terreno E, Wirth M, Strijkers GJ, Nicolay K, Gabor F. Surface modification of PLGA nanospheres with Gd-DTPA and Gd-DOTA for high-relaxivity MRI contrast agents. *Biomaterials*. 2010; 31:8716–8723.10.1016/j.biomaterials.2010.07.095 [PubMed: 20797782]
33. Hadjipanayis CG, Machaidze R, Kaluzova M, Wang L, Schuette AJ, Chen H, Wu X, Mao H. EGFRvIII antibody-conjugated iron oxide nanoparticles for magnetic resonance imaging-guided convection-enhanced delivery and targeted therapy of glioblastoma. *Cancer research*. 2010; 70:6303–6312. 0008-5472.CAN-10-1022 [pii]. 10.1158/0008-5472.CAN-10-1022 [PubMed: 20647323]
34. Muldoon LL, Sandor M, Pinkston KE, Neuwelt EA. Imaging, distribution, and toxicity of superparamagnetic iron oxide magnetic resonance nanoparticles in the rat brain and intracerebral tumor. *Neurosurgery*. 2005; 57:785–796. discussion 785–796. [PubMed: 16239893]
35. Platt S, Nduom E, Kent M, Freeman C, Machaidze R, Kaluzova M, Wang L, Mao H, Hadjipanayis CG. Canine model of convection-enhanced delivery of cetuximab-conjugated iron-oxide

- nanoparticles monitored with magnetic resonance imaging. *Clinical neurosurgery*. 2012; 59:107–113.10.1227/NEU.0b013e31826989ef [PubMed: 22960522]
36. Jung CW, Jacobs P. Physical and chemical properties of superparamagnetic iron oxide MR contrast agents: ferumoxides, ferumoxtran, ferumoxsil. *Magnetic resonance imaging*. 1995; 13:661–674. [PubMed: 8569441]
 37. Prince MR, Zhang HL, Chabra SG, Jacobs P, Wang Y. A pilot investigation of new superparamagnetic iron oxide (ferumoxytol) as a contrast agent for cardiovascular MRI. *Journal of X-ray science and technology*. 2003; 11:231–240. [PubMed: 22388293]
 38. Nguyen BC, Stanford W, Thompson BH, Rossi NP, Kernstine KH, Kern JA, Robinson RA, Amorosa JK, Mammone JF, Outwater EK. Multicenter clinical trial of ultrasmall superparamagnetic iron oxide in the evaluation of mediastinal lymph nodes in patients with primary lung carcinoma. *Journal of magnetic resonance imaging : JMRI*. 1999; 10:468–473. [PubMed: 10508310]
 39. Ragheb RR, Kim D, Bandyopadhyay A, Chahboune H, Bulutoglu B, Ezaldein H, Criscione JM, Fahmy TM. Induced clustered nanoconfinement of superparamagnetic iron oxide in biodegradable nanoparticles enhances transverse relaxivity for targeted theranostics. *Magnetic resonance in medicine : official journal of the Society of Magnetic Resonance in Medicine/Society of Magnetic Resonance in Medicine*. 2013; 70:1748–1760.10.1002/mrm.24622
 40. Neeves KB, Lo CT, Foley CP, Saltzman WM, Olbricht WL. Fabrication and characterization of microfluidic probes for convection enhanced drug delivery. *Journal of controlled release : official journal of the Controlled Release Society*. 2006; 111:252–262.10.1016/j.jconrel.2005.11.018 [PubMed: 16476500]
 41. Weinstein JS, Varallyay CG, Dosa E, Gahramanov S, Hamilton B, Rooney WD, Muldoon LL, Neuwelt EA. Superparamagnetic iron oxide nanoparticles: diagnostic magnetic resonance imaging and potential therapeutic applications in neurooncology and central nervous system inflammatory pathologies, a review. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism*. 2010; 30:15–35.10.1038/jcbfm.2009.192
 42. Saleh A, Schroeter M, Ringelstein A, Hartung HP, Siebler M, Modder U, Jander S. Iron Oxide Particle-Enhanced MRI Suggests Variability of Brain Inflammation at Early Stages After Ischemic Stroke. *Stroke*. 2007; 38:2733–2737.10.1161/strokeaha.107.481788 [PubMed: 17717318]
 43. Dousset V, Delalande C, Ballarino L, Quesson B, Seilhan D, Coussemacq M, Thiaudiere E, Brochet B, Canioni P, Caille JM. In vivo macrophage activity imaging in the central nervous system detected by magnetic resonance. *Magnetic resonance in medicine : official journal of the Society of Magnetic Resonance in Medicine/Society of Magnetic Resonance in Medicine*. 1999; 41:329–333.
 44. Neuwelt EA, Varallyay CG, Manninger S, Solymosi D, Haluska M, Hunt MA, Nesbit G, Stevens A, Jerosch-Herold M, Jacobs PM, Hoffman JM. The potential of ferumoxytol nanoparticle magnetic resonance imaging, perfusion, and angiography in central nervous system malignancy: a pilot study. *Neurosurgery*. 2007; 60:601–611. discussion 611–602. 10.1227/01.NEU.0000255350.71700.37 [PubMed: 17415196]

Figure 1. Morphologic and MR Characterization of SPIO-Loaded Brain-Penetrating Nanoparticles

Oleic acid- and oleylamine-coated SPIO were loaded into PLGA brain-penetrating nanoparticles and evaluated using scanning electron microscope (SEM) (A). Diameter measurements for the nanoparticle population were obtained using ImageJ (B). Measurements of SPIO-loaded nanoparticles effect on water relaxation at two different magnetic field strengths (4.0 T and 9.4 T), indicate that these nanoparticles are not very efficient T_1 relaxation agents (C) but excellent T_2 relaxation agents (D). Moreover, these measurements indicate that there are no significant differences between the SPIO effects on water relaxation at the two magnetic fields investigated. The longitudinal r_1 and transverse r_2 relaxivities of these nanoparticles were calculated from the linear fit according to eq.1 and are indicated in Table 1. Note that at 4.0T, the minimum TE possible was 15ms (due to hardware limitations) and thus for relaxation rates $R_2 > 100\text{s}^{-1}$, the MR signal decreases to less than 25% of the maximum value and the measurement of the relaxation rates becomes unreliable.

Figure 2. Dose-dependent MR Hypointensity of SPIO-Loaded Brain-Penetrating Nanoparticles Immediately after CED of 20 μ L brain-penetrating nanoparticles loaded with either 0.02 mg (A), 0.1 mg (B) or 0.5 mg (C) SPIO, a series of spin-echo MR images with various TE were acquired at 4.0T. The hypointensity in right caudate is due to SPIO-loaded brain-penetrating nanoparticles. The volume of this region, V_d , increases when we increased the SPIO NP dose. The corresponding T_2 maps were also calculated for these three doses (D–F). The spin-echo images (A–C) show an excellent agreement with the corresponding T_2 maps (D–F). However, the estimation of V_d depends on both the threshold used to define it and the value of TE used (Supplementary Figure 1). Moreover, using the T_2 maps to estimate V_d (defined in this case as the volume for which $T_2 < 80$ ms and indicated at the bottom right side of each T_2 map) gives similar estimations as when using the spin-echo images at higher echo times (TE > 80ms), where the MR contrast is maximized by longer TE.

Figure 3. MR Images at 4.0T Showing Gradual Loss of SPIO-Loaded Brain-Penetrating Nanoparticles with Time

Examples of MR images from the same animal scanned at various time points between 0 and 28 days (indicated below each image) following CED of 20 μ L of brain-penetrating nanoparticles containing 0.5mg total oleic acid- and oleylamine-coated SPIO (A–F). The hypointensity in right caudate is due to SPIO-loaded brain-penetrating nanoparticles. The results indicate a slow decrease of the SPIO volume V_d over the time period investigated (Supplementary Figure 3). An exponential fit of V_d/V_i as function of time for this animal indicate a lifetime $\tau = 89 \pm 9$ days according to eq.2.

Figure 4. Histological Examination Rat Brain One Month Following CED of SPIO-Loaded Brain-Penetrating Nanoparticles

After imaging experiments, the rat treated with 20 μ L of brain-penetrating nanoparticles containing 0.5 mg total oleic acid- and oleylamine-coated SPIO was sacrificed, brain harvested, and evaluated under H&E at low (A), medium (B), and high (C) magnification. Scale bars as indicated in panels; findings as described in text.

Table 1
Longitudinal (r_1) and transverse (r_2) relaxivities for SPIO nanoparticles at 9.4T and 4.0T

The relaxivities were obtained from the slope of the linear fit of the relaxation rates R_1 and R_2 at various SPIO NP concentrations, according to Eq.1, as shown in Figure 1 C and 1D.

Field	r_1 ($s^{-1} \text{ mg}^{-1} \text{ ml}$)	r_2 ($s^{-1} \text{ mg}^{-1} \text{ ml}$)
9.4 T	0.003 ± 0.002	236 ± 10
4.0 T	0.006 ± 0.005	202 ± 21