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Imaging the intratumoral-peritumoral extracellular pH gradient of gliomas

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Abstract

Solid tumors have acidic extracellular pH (pH_e) but near neutral intracellular pH (pH_i). Because acidic pH_e milieu is conducive to tumor growth and builds resistance to therapy, simultaneous mapping of pH_e inside and outside the tumor (i.e., intratumoral-peritumoral pH_e gradient) fulfills an important need in cancer imaging. We used **B**iosensor **I**maging of **R**edundant **D**eviation in **S**hifts (BIRDS), which utilizes shifts of nonexchangeable protons from macrocyclic chelates (e.g., 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrakis(methylene phosphonate) or DOTP⁸⁻) complexed with paramagnetic thulium (Tm^{3+}) ion, to generate in vivo pH_e maps in rat brains bearing 9L and RG2 tumors. Upon TmDOTP⁵⁻ infusion, MRI identified the tumor boundary by enhanced water transverse relaxation and BIRDS allowed imaging of intratumoral-peritumoral pH_e gradients. The pH_e measured by BIRDS was compared with pH_i measured with ³¹P-MRS. In normal tissue pH_e was similar to pH_i , but inside the tumor pH_e was lower than pH_i . While the intratumoral pH_e was acidic for both tumor types, peritumoral pH_e varied with tumor type. The intratumoral-peritumoral pH_e gradient was much larger for 9L than RG2 tumors, because in RG2 tumors acidic pH_e was found in distal peritumoral regions. Increased presence of Ki-67 positive cells beyond the RG2 tumor border suggested that RG2 was more invasive than 9L tumor. These results indicate that extensive acidic pH_e beyond the tumor boundary correlates with tumor cell invasion. In summary, BIRDS has sensitivity to map in vivo intratumoral-peritumoral pH_e gradient, thereby creating preclinical applications in monitoring cancer therapeutic responses (e.g., with pH_e -altering drugs).

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AUTHOR CONTRIBUTIONS

DC, YH, FH designed research. DC, YH, JUR, HMDF, CJ, FH performed research. DC, YH, JUR, FH analyzed data. DC, YH, JUR, DLR, FH wrote paper.

DISCLOSURE/CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

Keywords

metastasis; CEST; magnetic susceptibility; permeability; extravasation; SPIO

INTRODUCTION

Glioblastoma multiforme (GBMs) are the most lethal of brain cancers. In solid tumors extracellular pH (pH_e) is more acidic than intracellular pH (pH_i) which is neutral to alkaline (1). An acidic pH_e seems to favor tumor growth and metastasis by activating matrix metalloproteases and cathepsins (2). Rapidly growing tumor cells possess elevated rates of glucose uptake but reduced rates of oxidative phosphorylation (i.e., Warburg effect), thereby causing acidity in the extracellular space (3). Since acidic pH_e milieu is conducive to tumor cell propagation and builds resistance to therapy (4), imaging the intratumoral and peritumoral gradient of pH_e is an important, but yet unmet need in cancer imaging (5).

It has been shown that the acidic tumor microenvironment is related to the size and altered vascularity of tumors, which in turn stimulates hypoxia (6,7). Hypoxia induces gene expression changes that result in increased glycolysis and accumulation of carboxylic and lactic acids in the extracellular microenvironment. However, hypoxia is not the only contributor to acidification. Poor vascularity also hinders the elimination of protons and lactic acid contributing to lower pH_e . Low pH_e increases metastatic potential, angiogenesis, induction of genomic instability, and decreases immune function (8–11). Low pH_e also leads to resistance to radiotherapy and certain forms of chemotherapy (12). Since proton movement across the cell membrane is coupled to movement of other ions, pH_e is an important readout (13). Thus quantitative and simultaneous mapping of pH_e inside and outside the tumor (i.e., intratumoral-peritumoral pH_e gradient) can be useful for tumor localization and following response to cancer therapy (14).

Non-invasive magnetic resonance imaging (MRI) and spectroscopy (MRS) methods are mainstays for clinical cancer imaging (15). Smart MRI contrast agents enable physiochemical parameters like pH_e to be measured with lanthanide III ion (Ln^{3+}) derivatives of 1,4,7,10-tetraazacyclododecane (cyclen) (16), where most agents are built on the 1,4,7,10-tetraacetic acid (DOTA) backbone, similar to FDA-approved Gd^{3+} agents. While non- Gd^{3+} agents have yet to be tested in humans, molecular imaging with MRI has been demonstrated with a variety of DOTA-based macrocyclics in preclinical studies (17): relaxation-based methods and **C**hemical **E**xchange **S**aturation **T**ransfer (CEST). The relaxation-based methods rely on the paramagnetic effect induced by the Ln^{3+} ion on inner sphere of water protons, whereas CEST detects exchange between bulk water protons and an inner sphere of bound water that is shifted by the paramagnetic Ln^{3+} ion or exchangeable protons on the ligand (e.g., -OH or - NH_x).

Recently, we established a method that combines high spatial resolution MRI with high molecular specificity MRS into a 3D chemical shift imaging (CSI) platform called **B**iosensor **I**maging of **R**edundant **D**eviation in **S**hifts (BIRDS), where paramagnetically-shifted nonexchangeable protons (i.e., - CH_y) on DOTA-based macrocyclic complexes are directly detected (18–24). Longitudinal (T_1) and transverse (T_2) relaxation times of non-

exchangeable protons on BIRDS agents being very short (in ms range), allow high-resolution and high-speed CSI (18). BIRDS agents, similar to clinically-used DOTA, could potentially be translated across a range of magnetic fields because the T_2/T_1 ratio remains high and the proton shift sensitivities to the molecular event in the environment are largely unaffected. The readout of the physicochemical environment does not depend on diffusion or blood flow and BIRDS signals can be detected beyond MRI contrast (i.e., we see BIRDS signals when the MRI images are darkened by T_2 shortening) (18,19,21,22). Moreover, although the distribution of BIRDS agents may depend on vessel permeability (e.g. normal versus tumor tissue), the shift-based reporting is independent of agent's dose. Previously we showed that DOTA containing phosphonate agents (e.g., TmDOTP⁵⁻ and TmDOTA-4AmP⁵⁻) can provide pH_e maps with BIRDS in vivo (18,19).

In this study we used BIRDS to measure pH_e in the brains of 9L and RG2 tumor-bearing rats. We used TmDOTP⁵⁻ because of its commercial availability, but other BIRDS agents can also be used. Specifically, our goal was to see whether BIRDS can help determine if tumor invasiveness can be mapped by the intratumoral-peritumoral pH_e gradient, defined as the absolute pH_e difference between values inside and outside the tumor.

MATERIALS and METHODS

All experiments were performed according to NIH guidelines. Yale University's animal care and user committee (IACUC) approved the protocol. All scans were conducted on an 11.7T Agilent (Santa Clara, CA) horizontal-bore spectrometer with a bore size of 21 cm and maximum gradient strength of 400 mT/m, using ¹H and ³¹P surface radio-frequency (RF) probes (each 1.4 cm diameter).

Animal preparation for rats bearing 9L and RG2 tumors

Adult male Fischer 344 rats (200–250 g; n = 29) were obtained from Yale University vendors and maintained according to approved animal care protocols. RG2 (n = 16) and 9L (n = 13) tumors were studied (25). Rats were anesthetized with isoflurane (2–3%) and positioned in a stereotaxic instrument with the incisor bar set at 3.0 mm below the interaural line. RG2 and 9L cells at high confluence were harvested, washed, and suspended in appropriate media. Intrathalamic injections were made with a 25 µL Hamilton syringe, fitted with a 26 gauge beveled needle, into the right thalamus at coordinates 3 mm to the right from bregma and 3 mm ventral to the dura. A 5 µL volume of the cell suspension was injected over the course of 5 minutes and the needle was left in place for an additional 5 minutes before slow withdrawing. After injection the scalp was closed, treated with antibiotic, and non-steroidal anti-inflammatory drug (meloxicam, 1 mg/kg) was injected subcutaneously to prevent pain and inflammation.

On the day of the scanning, rats were anesthetized with isoflurane (<2%), tracheotomized and artificially ventilated (70% N₂O/30% O₂). A femoral vein was cannulated (PE-10) to administer TmDOTP⁵⁻ (0.4 mmol/kg). A femoral artery was cannulated (PE-50) for monitoring of physiological parameters (pCO₂, pO₂, pH, blood pressure) throughout the experiment. An intraperitoneal line was inserted for administration of α-chloralose (40 mg/kg/hr). α-chloralose provides stable physiology over long periods for terminal rat

experiments. However for longitudinal experiments isoflurane would be more ideal, but the exposure duration has to be kept short since vasomotion effects are induced by prolonged exposure of this anesthetic. Because the body temperature decreases in animals under anesthesia, we used a water-heating pad to maintain the body temperature in the physiological range (36–37 °C).

The anesthetized rats were prepared with renal ligation (18,26) to maintain a high TmDOTP⁵⁻ concentration gradient between the blood and extracellular space. Since renal ligation stops clearance of the agent, with slow infusion (~0.5 mL/hr) of the agent (19), the agent concentration builds up in the microvasculature sufficiently high enough for slow extravasation, which subsequently leads to agent accumulation in the extracellular space. Based on prior observations in rats without renal ligation (27), we expect higher agent accumulation in tumors (vs. normal tissue) because of porous immature blood vessels. Fenestrated vessels in circumventricular organ of normal brain may also play a role in agent accumulation in extracellular space (28–30). Previous ³¹P MRS experiments in normal rats showed that brain pH with and without renal ligation are similar (18,26). Ventilation was adjusted to maintain normal physiology. Body temperature was measured with a rectal temperature probe and no further adjustments in the water-heating pad were made for the entire duration of the experiment (~2 hrs). After experiments some rats were perfused and their brains were harvested for histopathological assessments.

Given that relaxation rates of agents like TmDOTP⁵⁻ or GdDOTA-4AmP⁵⁻ are possibly pH-dependent (18,23,27), we directly measured the TmDOTP⁵⁻ concentrations in normal and tumor tissues. A few rats (n = 4) were euthanized with focused microwave irradiation (31) and brain tissue (i.e., normal and tumor) was removed, weighed, and homogenized for agent concentration analysis ex vivo by ¹H NMR. Blood plasma samples were also collected to measure the agent in the plasma for correction of agent concentration in the tissue using tissue-plasma volumes (32). NMR solutions of each organ suspensions were prepared and 0.1 mM TmDOTMA⁻ was used as internal reference. NMR spectra were acquired using standard pulse-acquire experiments on an 11.7T vertical bore magnet using short TR and resonance intensities of H6 proton of TmDOTP⁵⁻ were measured. Final tissue concentrations of TmDOTP⁵⁻ were calculated after reference and plasma corrections.

Histological comparisons between 9L and RG2 tumors

Rats used for histochemical analysis were perfuse-fixed with 4% paraformaldehyde and embedded in paraffin. Brain tissue sections (4 µm) were stained with hematoxylin and eosin (H&E) for determination of tissue structure followed by immunohistochemical stains for monocarboxylate transporter 4 (MCT4), glial fibrillary acidic protein (GFAP) and Ki-67. Immunostaining was performed as previously described (33). Primary antibody diluted in 0.1% BSA/PBS was applied for 90 min at room temperature for GFAP (1:500) (DAKO, Z0334) and MCT4 (1:50) (H-90, Santa Cruz Biotechnology, sc-50329) and overnight at 4 °C for Ki-67 (1:100, Abcam, ab66155). The sections were incubated with a goat anti-rabbit secondary antibody (1:500, Pierce, 31460) for 60 minutes followed by incubation with metal enhanced 3,3-diaminobenzidine (DAB; Life technologies, 34065) for 10 min. Expression of various markers was quantified by evaluating the amount of DAB staining. The percentage

of the positive stained cells (PP) in each section was estimated by manually counting the cells as observed under $\times 50$ magnification and graded in a scale ranging from 0–100%. The DAB color intensity (I_{DAB}) was graded as non-staining (0), faint (1), medium (2) and intense (3). The staining score (SS) representing the expression of the markers was defined as follows

$$SS = I_{DAB} * PP \quad (1)$$

BIRDS for pH_e measurements

Prior to BIRDS experiments, spin-echo MRI datasets were obtained. 11 coronal slices of 128×128 resolution and 1 mm thickness were acquired using a field of view (FOV) of 25×25 mm², a recycle time (TR) of 6s and twelve different echo time (TE) values from 10 to 120 ms. Transverse relaxation time (T_2) and rate (R_2) maps were obtained in Matlab (MathWorks, Inc., Natick, MA) by fitting the absolute MRI intensity versus TE to a single exponential function (34). Following TmDOTP⁵⁻ infusion, the same MRI data were acquired to compare the T_2 changes induced by the paramagnetic agent (27).

The in vivo CSI acquisition for BIRDS was optimized for a 3D sampling of 1 μ L effective resolution, where the effective volume of a voxel was estimated by calculating the point spread function for gaussian-weighted spherical encoding (21,35). A dual-banded refocused 90° Shinnar-Le Roux (SLR) RF pulse of 35 kHz bandwidth and 90 kHz separation with 205 μ s duration was used for selective excitation of the H2/H3 and H6 protons of TmDOTP⁵⁻. The datasets were acquired with a TR of 5 ms and a FOV of $25 \times 25 \times 25$ mm³. The phase encode gradient duration was 160 μ s, the spectral window was 250000 Hz and the acquisition time was 4.1 ms. The ¹H spectrum for each encoding step was line broadened (300 Hz), phased (zero order), and baseline corrected (first order). The pH was calculated from the chemical shifts of the H2, H3, and H6 protons of TmDOTP⁵⁻ (δ_2 , δ_3 and δ_6) according to

$$pH = a_0 + \sum_{k=2,3,6} a_1^k \delta_k + \sum_{k=2,3,6} \sum_{j=2,3,6} a_2^{kj} \delta_k \delta_j \quad (2)$$

where the coefficients a_0 , a_1^k and a_2^{kj} were calculated from linear least-squares fit of pH as a function of chemical shifts δ_2 , δ_3 and δ_6 , as previously described (18). The pH readout by eq. 2 is unaffected by TmDOTP⁵⁻ concentration below 10 mM, which covers the TmDOTP⁵⁻ concentration range we observe in vivo (18,26). Within the pH range of 6.6–7.6, the changes in the chemical shift for the H2, H3, and H6 protons at 35 °C are 4.3, 3.4 and 4.3 ppm, respectively.

³¹P-MRS for pH_i measurements

Prior to BIRDS experiments, 6 glioma-bearing rats underwent single voxel ³¹P-MRS acquisitions for intratumoral pH_i and peritumoral pH_i measurements, where the localized volumes were determined by the tumor size. ³¹P spectra were acquired with ISIS localization (36) using a 1000 μ s adiabatic half-passage pulse and the following acquisition

parameters: TR = 16 s; number of averages = 96; dummy scans = 16. The pH_i was calculated by

$$pH_i = pK_a + \log \left(\frac{\Delta\delta - \delta_1}{\delta_2 - \Delta\delta} \right) \quad (3)$$

where δ is the chemical shift difference between inorganic phosphate (P_i) and phosphocreatine (PCr) resonances, $\delta_1 = 3.23$ ppm and $\delta_2 = 5.70$ ppm are the corresponding chemical shift differences at low and high pH, respectively (35), and $pK_a = 6.915 \pm 0.002$ was calculated by linear least-squares regression of P_i -PCr data for the different conditions.

Analysis of pH_e maps

Regional variations of pH_e measured by BIRDS were assessed for voxels inside the tumor, at the tumor margin, and well outside the tumor. The tumor margin was defined by MRI contrast. Intratumoral and peritumoral voxels were defined as all voxels inside and outside the tumor boundary defined by MRI respectively. The voxels at the tumor margin were defined as one voxel that were either partly inside or outside of the tumor (i.e., right on the tumor boundary defined by MRI contrast). A parameter f that represents the fraction of voxels with pH values smaller than a threshold value pH_{th} was calculated and a sigmoidal function (describing a cumulative distribution) was used to fit f versus pH_{th} according to the equation:

$$f(pH < pH_{th}) = \frac{1}{1 + e^{-4S_{pH}(pH_{th} - pH_m)}} \quad (4)$$

where pH_m represents the distribution midpoints (given by pH_{th} value for which $f = 0.5$) while S_{pH} represents the slope of the sigmoidal curve at $pH_{th} = pH_m$.

Statistics

All staining (eq. 1) and pH_e (eqs. 2 and 3) results were expressed as mean $\pm$ standard deviation (SD) and comparisons were assessed by Student's t test with two tails. Significance between pH_m measured intratumorally, within the tumor margin and peritumorally (eq. 4) were assessed by Student's t test, where p-values less than 0.05 were considered to be significant.

RESULTS

Figure 1 shows comparison of histological stains for 9L and RG2 tumors. At macroscopic scale the H&E staining of both tumors showed a greater extent of hematoxylin staining (i.e., blue pigmentation) in the parenchyma (vs. parenchyma of normal brain tissue), which at microscopic scale represented the tumoral mass within the inoculation zone (Figure 1A). The tumors were stained (eq. 1) for markers of proliferation (Ki-67), gliosis (GFAP) and glycolysis/lactate transport (MCT4). Ki-67 (Figure 1B) and MCT4 (Figure 1D) showed nuclear and membrane localization, respectively. Higher expression of Ki-67 (Figure 1B) and GFAP (Figure 1C) was detected in cells beyond the RG2 tumor boundary. Increased expression of MCT4 and GFAP was observed in RG2 tumor when compared to 9L, and in

both 9L and RG2 tumors cells migrating from the tumor boundary showed MCT4 positive cells. Examples of immunohistochemistry images for Ki-67, GFAP, and MCT4 in the same rat are shown in Figure S1, which are presented at different magnifications indicating locations of the images.

Figure S2 shows that MRI could identify the tumor with T_2 contrast upon TmDOTP⁵⁻ infusion, similar to Gd³⁺-induced longitudinal relaxation time (T_1) contrast. Due to the Tm³⁺-induced paramagnetic effect, tumors could be identified either by darkening caused by T_2 decrease (Figure S2A) or R_2 enhancement (Figure S2B) within intratumoral regions in relation to peritumoral regions. The TmDOTP⁵⁻ induced contrast in tumoral tissue is likely due to increased tumor vascular permeability allowing more TmDOTP⁵⁻ in the extracellular space of tumors compared to neighboring tissue.

Figure 2A and 2B show representative BIRDS data from 9L and RG2 bearing rats, respectively. The intratumoral regions of both tumors were localized similarly by T_2 -based contrast. The intensities of TmDOTP⁵⁻ protons were somewhat variable across different regions, with muscle signals being usually larger than those originating from the brain (Figure 2Bii). Since TmDOTP⁵⁻ clearance rates from and permeability into various tissues are likely to be different, in some cases muscle and brain (i.e., both normal and tumor tissue) signal intensities were quite similar (Figure 2Aii). While TmDOTP⁵⁻ proton intensities varied across brain regions, only the shift variations of H₂, H₃, and H₆ peaks allowed pH_e to be determined independent of the agent concentration using eq. 2, thus enabling pH_e maps to be compared across all voxels both within and beyond the tumor boundary. The intratumoral pH_e values were distinctly lower than peritumoral pH_e values. For example, in the 9L and RG2 tumors shown in Figure 2A and 2B the intratumoral pH_e values were 6.8 ± 0.1 and 6.7 ± 0.1 , respectively, whereas a large majority of voxels in the peritumoral regions had pH_e values of 7.2 or higher. But the brains with RG2 tumor showed patterns of diffuse pH_e reductions several mm beyond the tumor boundary. In the brain with RG2 tumor shown the pH_e values were quite similar in intratumoral regions vs. regions immediately adjacent to the tumor boundary, whereas in the brain with 9L tumor shown the pH_e values were distinctly smaller in intratumoral regions vs. regions immediately adjacent to the tumor boundary.

Figure 3 shows comparison of pH_e measured by BIRDS and pH_i measured using ³¹P-MRS from the same animals bearing small gliomas. For BIRDS and ³¹P-MRS, the pH-induced shifts are ~4 and ~1 ppm/pH unit, respectively, with measurement error <0.01 pH units (18). ³¹P spectra inside a tumor (Figure 3A) and outside a tumor (Figure 3B) show pH_i values that were neutral in both locations. In the same rat, after TmDOTP⁵⁻ infusion, the BIRDS data show lower pH_e values inside vs. outside the tumor (Figure 3C). Overall, there was good agreement between pH_i and pH_e in normal tissue outside the tumor, whereas inside the tumor pH_e was significantly lower than pH_i (Figure 3D).

BIRDS had sufficient sensitivity to distinguish different sizes of 9L (Figure 4) and RG2 (Figure 5) tumors. However, in case of small tumors (1–2 mm diameter) the partial contribution from non-tumor tissue (based on tumor boundary defined by T_2 -based contrast) could become significant. To minimize this contribution, for intratumoral pH_e calculation we used only the voxels with a partial volume of more than 50% occupied by tumor tissue.

Thus, we estimated the error in pH_e gradient between tumor and non-tumor tissue assuming 50% partial volume for voxels positioned at the tumor boundary and a nominal pH_e gradient of 0.1 within each boundary voxel. Since for small tumors (6 voxels) all voxels touch the boundary and experience partial volume effects, then the calculated error in pH_e gradient reported would be less than 0.05 units. In contrast, however, for larger tumors (40–50 voxels) only about 25% of the voxels touch the boundary and experience partial volume effects. Therefore, in larger tumors, the error in pH_e gradient estimation would be less than 0.013 units. Since this suggests that the error in pH_e gradient between the tumor and its boundary scales inversely with the tumor size, we analyzed the larger 9L and RG2 tumors to find some differences in terms of intratumoral vs. peritumoral pH_e measurements. Overall, the intratumoral pH_e values of both tumor types became more acidic with tumor growth and changes in the peritumoral pH_e depended on the tumor type (i.e., more acidic peritumoral pH_e for tumors that invade).

For 9L tumors (Figure 4), the distributions of peritumoral pH_e values in a small (Figure 4A) and a large (Figure 4B) tumor were overlapping (e.g., 7.2–7.6), suggesting that peritumoral pH_e is not significantly affected by 9L tumor growth. The distribution of intratumoral pH_e values in a large tumor (e.g., 6.7–7.2) was significantly shifted into acidic ranges compared to the intratumoral pH_e values in a small tumor (e.g., 7.0–7.2), suggesting that intratumoral pH_e becomes more acidic with 9L tumor growth.

For RG2 tumors (Figure 5), the distributions of peritumoral pH_e values in a small (Figure 5A) and a large (Figure 5B) tumor were overlapping but the spread was much larger than observed for 9L tumors (e.g., 6.8–7.6), suggesting that peritumoral pH_e is already quite acidic in the earliest stages of RG2 tumor growth. The distribution of intratumoral pH_e values in a large tumor (e.g., 6.6–7.2) was significantly shifted into acidic ranges compared to the intratumoral pH_e values in a small tumor (e.g., 6.9–7.2), suggesting that intratumoral pH_e becomes more acidic with RG2 tumor growth.

The intratumoral-peritumoral pH_e gradients (Figure 6) measured by BIRDS were compared quantitatively. The sigmoidal relationships between pH_e thresholds and percentage of voxels with pH_e below these thresholds were represented by eq. 4. For brains with 9L tumors (Figure 6A) ~80% of intratumoral voxels had pH_e lower than 7.0 (with values as low as 6.6) and ~80% of peritumoral voxels had pH_e lower than 7.4 (with values as low as 7.1), whereas pH_e trends for voxels within the tumor boundary were in between these two other trends. For brains with RG2 tumors (Figure 6B) ~80% of intratumoral voxels had pH_e lower than 7.0 (with values as low as 6.6) and ~80% of peritumoral voxels had pH_e lower than 7.3 (with values as low as 6.8), whereas pH_e trends for voxels within the tumor boundary nearly matched pH_e trends for peritumoral voxels. Hence, for 9L tumors the peritumoral pH_e was clearly separated from intratumoral pH_e and pH_e within the tumor boundary (Figure 6C). But in RG2 tumors the peritumoral pH_e was only clearly separated from intratumoral pH_e because peritumoral pH_e was quite similar to pH_e within the tumor boundary (Figure 6C). These results indicate a significantly steeper intratumoral-peritumoral pH_e gradient for brains with 9L tumors compared to brains with RG2 tumors (i.e., ~0.4 vs. ~0.2; Figure 6C). The histograms summarizing the results in terms of pH_e distribution measured by BIRDS for intratumoral, margin, and peritumoral regions for 9L and RG2 tumors are shown in

Figure S3. The most probable pH_e values and the full width at half maximum (FWHM) obtained from the Gaussian fit are given in Table 1. As expected, for both 9L and RG2 tumors, the most probable pH_e values obtained from the Gaussian fit of the pH_e distribution (Figure S3 and Table 1) were the same (within the estimated error of the fit) as the distribution midpoints pH_m obtained from the Sigmoidal fit (Figure 6), indicating that both types of analyses gave the same result.

DISCUSSION

Because acidic pH_e is believed to facilitate tumor cell invasion (2), quantitating pH_e could have significant implications for diagnosis of tumor invasiveness and response to therapy (14,37). Since most MRI-based molecular imaging methods focus on the intratumoral pH_e (27,38,39), the need for mapping the intratumoral-peritumoral pH_e gradient has escaped attention. Thus we used BIRDS to measure intratumoral-peritumoral pH_e gradient in rat brains bearing 9L and RG2 tumors to assess how this parameter changes with tumor invasiveness.

Pros and cons of molecular imaging with BIRDS

BIRDS is a molecular imaging platform that detects paramagnetically-shifted nonexchangeable protons on the agent as opposed to water proton relaxation, which is the norm in molecular imaging with MRI. Because a paramagnetic CEST agent also has nonexchangeable protons, these CEST agents also show BIRDS properties and allow dual modality with the same agent (20,23). BIRDS is compatible with superparamagnetic iron oxide (SPIO) and the pH_e readouts from BIRDS are unaffected by high dose SPIO (40), because the linewidths of TmDOTP^{5-} resonances in rat brain are already large (1–2 ppm) and they do not change significantly after SPIO addition. Therefore it should be possible to apply SPIO nanoparticles to monitor drug delivery by MRI and then use BIRDS to monitor the physicochemical effects (e.g., pH_e) of the drugs delivered.

Signal of agents like TmDOTP^{5-} and TmDOTMA^- can be observed in the brain of rats that have undergone renal ligation even after several hours following the start of the infusion (19), suggesting slow clearance from the extracellular space. We previously measured the presence of TmDOTP^{5-} in the cerebral spinal fluid (26). We proposed that fenestrated vessels of circumventricular organs may also provide an additional route for these agents to gain access to the extracellular space, because fenestrated vessels in this location are present even in the adult brain (28–30). Additionally, using ^1H MRS, at the end of 2 hours of infusion, we measured TmDOTP^{5-} concentration in blood to be ~3 mM, while in extracted tissues from normal and tumor regions the TmDOTP^{5-} concentrations were ~0.2 and ~0.5 mM, respectively. These signals contain ~13% contribution from blood (3% partial volume) and ~87% from extracellular space (20% partial volume) (18,19,26). Due to lack of reports on the volumes of extracellular and intravascular spaces in brain tumors, we assumed that the fractions of these compartments inside and outside tumors are the same. Thus TmDOTP^{5-} in extracellular space of normal and tumor tissues was ~0.5 mM and ~2 mM, respectively.

Protonation/deprotonation of phosphonate groups of DOTP⁸⁻ or DOTA-4AmP⁸⁻ is fast on the NMR timescale, which can be observed either by the pH-dependent variations of the H₂, H₃ and H₆ chemical shifts with BIRDS (18) or relaxation MRI (27). Thus, protonation/deprotonation of TmDOTP⁵⁻ or other agents such as GdDOTA-4AmP⁵⁻ is not diffusion limited in free solution, but it might be affected in normal or tumor tissue due to different water diffusion environments. However even for regions with restricted (slow) water diffusion, we and others expect that protonation/deprotonation equilibrium is reached rapidly because TmDOTP⁵⁻ (or GdDOTA-4AmP⁵⁻) is a small molecule and is dilute in an otherwise aqueous medium (18,27). Moreover in vitro BIRDS measurements with large temperature variations do not affect the pH readout capability of DOTP⁸⁻ (or DOTA-4AmP⁸⁻) (18,23), suggesting that the pH_e measured with such probes represent protonation/deprotonation equilibrium over the observed diffusion range in vivo. However future studies with ADC and high-resolution BIRDS may indicate possible correlation (or lack thereof) between pH_e and water diffusion.

The paramagnetic effect of TmDOTP⁵⁻ on water protons provides sufficient T₂ contrast for identification of the intratumoral volume, similar to enhanced T₁ contrast with Gd³⁺ agents for tumor detection (26,27). As suggested by Martinez et al (27) and others (26), greater extent of these agents' extravasation across porous tumor blood vessels is responsible for the superior MRI contrast between tumor and surrounding tissue (Figure S2). In addition, detection of the paramagnetically-shifted TmDOTP⁵⁻ protons with BIRDS allows physicochemical imaging of the brain (Figure 2).

While the current study used TmDOTP⁵⁻ for pH detection with BIRDS, in future studies methyl groups can be added in select regions of the ligand to improve sensitivity, thereby allowing reduced CSI voxel sizes and/or decreasing the amount of agent needed. At present, 1 μ L voxel size in 3D CSI of whole brain is acquired in ~10 minutes with a minimum of ~0.2 mmol/kg dose using methylated agents (e.g., TmDOTMA⁻) for nominal SNR (21,22). In addition, preliminary results in our laboratory towards avoiding surgical intervention used to raise plasma levels of the agent (18,19), show that it is possible to co-infuse intravenously the agent with drugs that temporarily inhibit renal excretion (e.g., probenecid is used in humans to raise plasma concentration of drugs) (41). The co-infusion of the agent and probenecid should also lower the agent dose and allow repeated scans for longitudinal studies.

Morphology of 9L and RG2 tumors

Syngenic rat brain tumor models are widely used in preclinical cancer research (25). 9L and RG2 are considered gliosarcoma and glioblastoma models, respectively. A distinct advantage of such syngeneic models is that they are grafted into fully immune competent rats. 9L tumor margins are sharply delineated with little obvious invasion into the contiguous normal brain. The RG2 glioma has a relatively more infiltrative growth with foci of local collective invasion (25). 9L and RG2 tumors have different metabolite profiles and differences in energy demand (42). Microvessel density and angiogenesis seem to be higher in RG2 when compared to 9L by histological studies, however MRI methods could not reveal these differences (43).

Higher Ki-67 staining was detected in cells beyond the RG2 tumor boundary (Figure 1B) in agreement with prior observations (44). In addition, RG2 showed significantly higher GFAP (Figure 1C) and MCT4 (Figure 1D) staining inside the tumor compared to 9L, while significantly higher GFAP staining was observed outside the tumor in RG2 when compared to 9L. These results collectively imply morphological differences between 9L and RG2, where RG2 tumor cells seem to invade beyond the tumor boundary more than 9L tumor cells.

pH_e mapping in tumors

Various factors contribute to the acidity of the extracellular space in peritumoral and intratumoral regions. It is generally believed that the Warburg effect mediates the acidosis within the tumor core, while beyond the tumor core inflammatory responses by lymphocytes and macrophage infiltrations could certainly affect pH_e. However, it should be noted that poor perfusion in tumors hampers the ability of the microenvironment to remove tumor-derived acid through diffusion into blood vessels. Thus, H⁺ ions and lactic acid generated as a result of tumoral Warburg effect can diffuse through interstitial space along the concentration gradient due to lack of effective clearance by blood flow (45).

The low intratumoral pH_e values in gliomas measured here are similar to prior MRI/MRS reports (27,46). We compared pH_e measured by BIRDS with pH_i measured by ³¹P-MRS. In both tumors, intratumoral pH_e from BIRDS was significantly lower (~6.9) compared to intratumoral pH_i from ³¹P-MRS (~7.4) and peritumoral pH_e approached peritumoral pH_i (Figure 3). In agreement with prior observations (1,14), our results indicate that the intratumoral pH_e-pH_i gradient is large, whereas the peritumoral pH_e-pH_i gradient is negligible. But the intratumoral-peritumoral pH_e gradient was much smaller for brains bearing RG2 vs. 9L tumors, indicating tumor invasion relates to diffuse areas of low pH_e (Figure 6).

A good example of pH_e measurement in brain tumors is by Martinez et al, where they used a pH-sensitive T₁ agent GdDOTA-4AmP⁵⁻ injected intravenously into rats with C6 tumors with a pH-insensitive T₂ agent DyDOTP⁵⁻ (27). The T₂ change caused by DyDOTP⁵⁻, similar to T₂ darkening we observed with TmDOTP⁵⁻ in this study (Figure S2), allowed [Gd³⁺] estimation to convert the T₁ change of GdDOTA-4AmP⁵⁻ into pH_e. Alternate MRI methods using CEST, with either diamagnetic or paramagnetic agents (47), provide relative estimates of intratumoral pH_e. Diamagnetic CEST has detected enhanced amide proton exchange (APT) in brain tumors (38,48), where the APT is related to pH_i. Diamagnetic CEST has been applied for other types of tumors with low intratumoral pH_e (39).

Since the focus in all previously reported pH-dependent maps with MRI and CEST methods was on intratumoral pH_e, the intratumoral-peritumoral pH_e gradient had not been quantitatively determined. Peritumoral pH_e mapping has not received attention in past studies, and hence, in the absence of such data it could be inherently assumed that peritumoral pH_e is close to normal tissue pH_e. In this study we tested this assumption in two different tumor types and we showed that the peritumoral pH_e of an aggressive tumor (RG2) is more acidic than normal tissue pH_e. The MRI-measured intratumoral pH_e reported by GdDOTA-4AmP⁵⁻ for C6 tumors (27) is in general agreement with BIRDS-measured

intratumoral pH_e reported by TmDOTP⁵⁻ for 9L and RG2 tumors. However in the present study we were able to compare the peritumoral pH_e with intratumoral pH_e . The ability to compare the intratumoral-peritumoral pH_e gradient may be an important distinction as the diffuse nature of pH_e reductions beyond the tumor boundary has been linked to tumor invasiveness (i.e., RG2 has higher extent of Ki-67 positive cells beyond the tumor boundary). These results are in good agreement with past studies, using primarily MRS techniques, that have shown that the intratumoral-peritumoral pH_e gradient varies with tumor type (for a recent review see (2)).

SUMMARY

Our results show that intratumoral-peritumoral pH_e gradient mapping by BIRDS can be used for high-resolution spatial evaluation of the tumor microenvironment. While acidic intratumoral pH_e is a target for various cancer drugs (49), future chemotherapies could be further targeted to the diffuse areas of low peritumoral pH_e because tumor cells invade (50).

Targeting the acidic pH_e of the tumor microenvironment provides several therapeutic options. The proton pump seems to be a successful target for inhibitor designs to change the intratumoral pH_e . For example, the V-ATPase inhibitor bafilomycin A1 inhibits tumor growth by increasing the intratumoral pH_e without perturbing the intratumoral pH_i (51). Another approach for increasing intratumoral pH_e is systemic buffering by sodium bicarbonate and/or trisodium citrate (52,53).

Overall, a combination of pH regulating drugs with other cancer treatments (e.g., targeting glycolysis, chemotherapy and/or radiation) might help to reduce the tumor growth or prevent metastasis. One such example is the bicarbonate approach combined with dichloroacetate, which increases pyruvate dehydrogenase flux (52). In any event, if we can spatially localize the regions of low pH_e , both within and beyond the tumor margin, we may detect tumor invasion, proliferation, angiogenesis, and response to therapy to determine the prognosis of the disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

APT	amide proton exchange
BIRDS	biosensor imaging of redundant deviation in shifts
CEST	chemical exchange saturation transfer
CSI	chemical shift imaging

DAB	diaminobenzidine
DOTP8-	1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrakis methylene phosphonate
FOV	field of view
FWHM	full width at half maximum
GFAP	glial fibrillary acidic protein
GBM	glioblastoma multiforme
H&E	hematoxylin and eosin
Pi	inorganic phosphate
MCT4	monocarboxylate transporter 4
PP	percentage of positive stained cells
PCr	phosphocreatine
RF	radio-frequency
SLR	Shinnar-Le Roux
SD	standard deviation
SS	staining score
SPIO	superparamagnetic iron oxide

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Figure 1.

Evaluation of 9L and RG2 tumors by immunohistochemistry. Examples of staining results for (A) H&E, (B) Ki-67, (C) GFAP, and (D) MCT4 in individual rats. Black and blue arrows show positive DAB staining outside and inside tumor boundary, respectively (scale bar = 100 μ m; red lines in **B** to **D** indicate tumor boundary) for 9L (top) and RG2 (middle) tumors. Averaged staining scores (eq. 1) inside and outside tumor boundary (bottom) for a group of rats (n = 3 for each tumor type) are shown in graphs. Ki-67 expression outside tumor core in RG2 was significantly higher than in 9L (*, $p < 0.031$); GFAP expression was higher in RG2 tumors than in 9L both inside and outside tumor boundary (#, $p < 0.025$ and 0.027, respectively). While MCT4 staining was higher in RG2 tumors than 9L tumors, the difference did not reach significance.

Figure 2.

Representative pH_e maps from BIRDS data of rats bearing (A) 9L and (B) RG2 tumors. In (i) the T_2 -weighted image shows the tumor localization identified by darkening (tumor = blue outline; brain = brown outline), which is likely due to increased tumor vascular permeability allowing more TmDOTP $^{5-}$ in the interstitial space of the tumor compared to neighboring non-tumor tissue (see Figure S2). In (ii) a portion of the 3D CSI data for the slice in (i) shows varying TmDOTP $^{5-}$ levels throughout the brain. In (iii) examples of spectra from intratumoral and peritumoral voxels. In (iv) quantitative maps of pH_e were obtained using multiple TmDOTP $^{5-}$ peaks and their respective pH sensitivities (eq. 2). (A) Specific voxels in a brain bearing 9L tumor shows significantly shifted TmDOTP $^{5-}$ peaks indicating different pH_e inside ($pH_e = 6.79$) than outside ($pH_e = 7.17$) the tumor boundary. The 9L tumor was identified by significantly lower pH_e within the tumor boundary. (B) Specific voxels in a brain bearing RG2 tumor shows significantly shifted TmDOTP $^{5-}$ peaks indicating different pH_e inside ($pH_e = 6.69$) than outside ($pH_e = 7.29$) the tumor boundary. The RG2 tumor was clearly identified by lower pH_e within the tumor boundary. While the most significantly low pH_e voxels were found inside the tumor margin, there were low pH_e voxels that were spread well beyond the tumor margin.

Figure 3.

Comparison of pH measured by ^{31}P -MRS (pH_i) and BIRDS (pH_e) in small gliomas (~ 2 mm). The spectroscopic data were sequentially acquired in the same rats, first with ^{31}P -MRS and then with BIRDS. The pH measured by ^{31}P -MRS was calculated from the PCr and P_i shifts (eq. 3), which reflects the intracellular compartment, whereas the pH measured by BIRDS (eq. 2) represents the extracellular compartment. (A) ^{31}P spectrum from a voxel localized inside the tumor showed a pH value of 7.43. (B) ^{31}P spectrum from a voxel localized outside the tumor (i.e., inside normal brain tissue) showed a pH value of 7.42. (C) MRI and BIRDS data from the same rat, where the pH inside and outside the tumor were approximately 7.0 and 7.4, respectively. The tumor is indicated with the blue outline, whereas the brain is shown with brown outline. (D) Averaged pH measured by ^{31}P -MRS (black bars) and BIRDS (white bars) across a group of rats ($n = 6$). Good agreement between pH_i (by ^{31}P -MRS) and pH_e (by BIRDS) in normal brain tissue ($p > 0.5$), whereas inside the tumor pH_i (by ^{31}P -MRS) was significantly higher than pH_e (by BIRDS). *Abbreviations:* PCr, phosphocreatine; P_i , inorganic phosphate.

Figure 4.

Representative BIRDS data from individual rats bearing 9L gliosarcomas of different sizes, shown in terms of pH_e maps and their respective histograms for values inside (intratumoral) and outside (peritumoral) the tumor. The MRI depiction of the tumor size is shown on the left (tumor = blue outline; brain = brown outline). **(A)** Data for a small 9L (~2 mm) shows that the pH_e were significantly separated for intratumoral voxels ($pH_e = 7.01 \pm 0.08$) and peritumoral voxels ($pH_e = 7.34 \pm 0.09$). **(B)** Data for a large 9L (~6 mm) shows also that the pH_e values were significantly separated for intratumoral voxels ($pH_e = 6.91 \pm 0.17$) and peritumoral voxels ($pH_e = 7.31 \pm 0.13$). The distributions of peritumoral pH_e values in small and large tumors were overlapping (e.g., 7.2–7.6). The distribution of intratumoral pH_e values in large tumor (e.g., 6.7–7.2) was significantly shifted into acidic ranges compared to the intratumoral pH_e values in small tumor (e.g., 7.0–7.2).

Figure 5.

Representative BIRDS data from individual rats bearing RG2 gliomablastomas of different sizes, shown in terms of pH_e maps and their respective histograms for values inside (intratumoral) and outside (peritumoral) the tumor. The MRI depiction of the tumor size is shown on the left (tumor = blue outline; brain = brown outline). **(A)** Data for a small RG2 (~2 mm) shows that the pH_e were significantly separated for intratumoral voxels ($pH_e = 6.84 \pm 0.22$) and peritumoral voxels ($pH_e = 7.31 \pm 0.16$). **(B)** Data for a large RG2 (~5 mm) shows also that the pH_e values were significantly separated for intratumoral voxels ($pH_e = 6.91 \pm 0.17$) and peritumoral voxels ($pH_e = 7.18 \pm 0.14$). The distributions of peritumoral pH_e values in small and large tumors were overlapping but the spread was quite large (e.g., 6.8–7.6). The distribution of intratumoral pH_e values in large tumor (e.g., 6.6–7.2) was significantly shifted into acidic ranges compared to the intratumoral pH_e values in small tumor (e.g., 6.9–7.2).

Figure 6.

The intratumoral-peritumoral pH_e gradient measured by BIRDS for 9L ($n = 13$) and RG2 ($n = 16$) tumors. Quantitative variations of pH_e were obtained for intratumoral voxels (red circles), voxels within the tumor margin (blue squares), and peritumoral voxels (black diamonds). In (A) and (B) the vertical axis is a parameter f that represents the fraction of voxels with pH_e values smaller than a threshold value (pH_{th}), which is indicated on the horizontal axis. Variations of pH_e for 9L and RG2 tumors show sigmoidal relationships for all voxels. The data points are the average, the error bars are the standard error, and the lines are the non-linear fit of f to eq. 4 for intratumoral voxels (red line), voxels within the tumor margin (blue line), and peritumoral voxels (black line). (A) For 9L tumors, pH_e values of the margin were significantly different from peritumoral and intratumoral pH_e values. (B) For RG2 tumors, pH_e values of the margin were not significantly different from peritumoral pH_e values, but significantly different from intratumoral pH_e values. (C) The overall pH_e differences between 9L and RG2 tumors are shown with regard to the distribution midpoint pH_m , which represents the pH_e at $f = 0.5$. Statistical group comparison using Student's t-test with two tails for pH_e shows that pH_m was significantly different for 9L ($p < 0.001$) for all three possible comparisons (intratumoral vs. margin, peritumoral vs. margin, and intratumoral vs. peritumoral), while for RG2 was significantly different ($p < 0.0001$) only for intratumoral vs. margin and intratumoral vs. peritumoral comparisons. When comparing pH_m values for 9L versus RG2, only the peritumoral regions showed significant differences ($p < 10^{-6}$).

Table 1

Most probable pH_e values and the FWHM obtained from the fit of the normal (Gaussian) distribution versus pH_e shown in Figure S3 for intratumoral, margin and peritumoral regions in 9L and RG2 tumors.

Region	9L		RG2	
	pH_e	FWHM	pH_e	FWHM
intratumoral	6.90 ± 0.01	0.38 ± 0.01	6.87 ± 0.01	0.44 ± 0.02
tumor margin	7.09 ± 0.01	0.41 ± 0.02	7.08 ± 0.01	0.50 ± 0.03
peritumoral	7.29 ± 0.01	0.32 ± 0.02	7.13 ± 0.01	0.35 ± 0.01