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Imaging chemical exchange saturation transfer (CEST) effects following tumor-selective acidification using lonidamine

Nevin McVicar\textsuperscript{a}, Alex X. Li\textsuperscript{b}, Susan O. Meakin\textsuperscript{b,c} and Robert Bartha\textsuperscript{a,b,*}

Increased lactate production through glycolysis in aerobic conditions is a hallmark of cancer. Some anticaner drugs have been designed to exploit elevated glycolysis in cancer cells. For example, lonidamine (LND) inhibits lactate transport, leading to intracellular acidification in cancer cells. Chemical exchange saturation transfer (CEST) is a novel MRI contrast mechanism that is dependent on intracellular pH. Amine and amide concentration-dependent detection (AACID) and apparent amide proton transfer (APT\textsuperscript{*}) represent two recently developed CEST contrast parameters that are sensitive to pH. The goal of this study was to compare the sensitivity of AACID and APT\textsuperscript{*} for the detection of tumor-selective acidification after LND injection. Using a 9.4-T MRI scanner, CEST data were acquired in mice approximately 14 days after the implantation of $10^5$ U87 human glioblastoma multiforme (GBM) cells in the brain, before and after the administration of LND (dose, 50 or 100 mg/kg). Significant dose-dependent LND-induced changes in the measured CEST parameters were detected in brain regions spatially correlated with implanted tumors. Importantly, no changes were observed in $T_1$- and $T_2$-weighted images acquired before and after LND treatment. The AACID and APT\textsuperscript{*} contrast measured before and after LND injection exhibited similar pH sensitivity. Interestingly, LND-induced contrast maps showed increased heterogeneity compared with pre-injection CEST maps. These results demonstrate that CEST contrast changes after the administration of LND could help to localize brain cancer and monitor tumor response to chemotherapy within 1 h of treatment. The LND CEST experiment uses an anticancer drug to induce a metabolic change detectable by endogenous MRI contrast, and therefore represents a unique cancer detection paradigm which differs from other current molecular imaging techniques that require the injection of an imaging contrast agent or tracer. Copyright © 2015 John Wiley & Sons, Ltd.

**Keywords:** Chemical exchange saturation transfer < Endogenous Contrast Methods < Methods and Engineering; Magnetization transfer (MT) < Endogenous Contrast Methods < Methods and Engineering; Animal model study < Cancer < Applications; Cellular and molecular cancer imaging < Cellular and molecular imaging < Applications

INTRODUCTION

Primary brain glioma is a rare form of cancer accounting for less than 2% of all cancers; however, it represents the second leading cause of death among neurological diseases in the USA (1,2). Glioblastoma multiforme (GBM) is the most lethal and most common form of glioma (2–4). The current standard of care of GBM tumors includes surgical resection of the primary tumor, followed by aggressive concurrent radiation and chemotherapy (2–4). Despite advances in therapeutic strategies, coupled with neurological imaging throughout treatment, mean GBM patient survival time is approximately 1 year after diagnosis (1–4). Over 90% of GBM tumors recur as a result of migratory cancer cells that evade treatment (4).

Currently, clinicians rely on standard anatomical $^1$H MRI to detect tumors and to monitor treatment response (2,4); yet, morphological changes caused by treatment are often difficult to detect and quantify, especially during radiation therapy when pseudo-progression markers may exist (3,5). Using anatomical MRI techniques, clinicians often require several weeks to conclude that a GBM tumor is unresponsive to therapy before prompting a change in treatment (4). As GBM tumors are among the most aggressive human tumors, there is a need to develop imaging techniques that are able to detect tumor response within days or even hours after initial treatment. For example, Zhou et al. (5) have shown recently that tumor response to radiation therapy (i.e. radiation necrosis) can be detected within 3 days of treatment using a novel $^1$H MRI contrast mechanism.

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**Abbreviations used:** AACID, amine and amide concentration-independent detection; APT*, apparent amide proton transfer; CEST, chemical exchange saturation transfer; CNR, contrast-to-noise ratio; ETL, echo train length; FSE, fast spin-echo; GBM, glioblastoma multiforme; H&E, hematoxylin and eosin; LND, lonidamine; MT, magnetization transfer; MTR asym, asymmetric magnetization transfer ratio; NOE, nuclear Overhauser effect; PBS, phosphate-buffered saline; pH, intracellular pH; RF, radiofrequency; ROI, region of interest; WASSR, water saturation shift referencing.
called chemical exchange saturation transfer (CEST). The ability to quantify tumor response immediately after therapy will enable clinicians to quickly tune and optimize therapy, improving patient outcome.

Malignant cancer cells undergo genetic mutations that result in increased aerobic glycolysis, known as the Warburg effect (6). The Warburg effect leads to higher lactic acid production, but cancer cells adaptively increase the expression of intracellular pH (pHi) regulators (e.g., ion transport channels and pH-i-buffering enzymes) to maintain a relatively alkaline pHi (7). Alkaline pHi enables increased proliferation and evasion of apoptosis by cancer cells (7). A class of chemotherapy agents target increased glycolysis in tumors by inhibiting key pHi buffers and/or glycolytic enzymes, such as hexokinase, or by blocking ion transport channels (6). Consequently, many chemotherapy agents cause changes in physiological properties, such as pHi, almost immediately after starting treatment (8–11). The monitoring of immediate tumor pHi response to metabolic stressors could provide a measure of tumor aggressiveness and predict tumor response to treatment (12). For example, Sagiyama et al. (3) recently used a pH-sensitive CEST MRI contrast to monitor tumor response to chemotherapy with temozolomide. It was found that CEST MRI contrast changed significantly in orthotopic GBM tumors, 7 days after a single course of temozolomide treatment, compared with untreated tumors. As no changes in tumor volume, cell density or apoptosis were observed in treated tumors, Sagiyama et al. hypothesized that the observed changes in CEST contrast may be attributed to a decrease in pHi caused by temozolomide.

The purpose of the current study was to evaluate the sensitivity of pH-dependent CEST contrast for the detection of changes in tumor pHi induced by a single dose of the drug lonidamine (LND), which has been used to treat cancer in Europe and Canada (10). LND is an anticancer agent that exploits the Warburg effect to target and destroy malignant cancer cells (6,10). More specifically, LND limits aerobic respiration in cancer cells by blocking pyruvate transport from the cytoplasm (13). As a result of the Warburg effect, cancer cells exhibit a greater reliance on pyruvate produced by glycolysis in the cytoplasm compared with normal cells. The inhibition of pyruvate transport impedes oxidative phosphorylation in tumors, leading to energy depletion (10,14). Furthermore, LND blocks lactate efflux from cells by inhibiting the monocarboxylic acid transporter (10). Cancer cells produce significantly more lactate than normal cells (6) and, consequently, we hypothesize that tumor-selective intracellular acidification occurs (10,11,14) following LND treatment that will produce CEST MRI contrast.

**THEORY**

CEST contrast is generated using protons associated with endogenous mobile proteins and peptides that are: (i) chemically shifted by δω from bulk water protons; and (ii) freely exchanging at a rate kex with bulk water protons. To generate CEST contrast, exchangeable protons associated with specific molecules can be saturated using a frequency-selective radiofrequency (RF) saturation pulse. The process of chemical exchange transfers saturated protons to the bulk water proton pool, leading to a decrease (darkening) in MR image intensity. CEST effects from amide protons resonating at δω = 3.50 ppm downfield (i.e., higher frequency) from bulk water protons (referenced to 0 ppm) or amine protons resonating at δω = 2.0 and δω = 2.5–2.75 ppm have been used by several groups to generate CEST contrast (15–19). Amide and amine proton CEST effects are pH dependent as their chemical exchange rates kex are base catalyzed (20). The majority of mobile proteins and peptides reside in cells, and therefore amide and amine CEST effects are considered to be pH dependent. Cerebral ischemia animal models have been used to demonstrate a local decrease in amide CEST effects at 3.50 ppm and increase in amine CEST effects at 2.5 ppm in regions of intracellular acidosis (15–17).

CEST effects may be observed using a z-spectrum. A z-spectrum is a plot of the normalized bulk water magnetization $M_{sat}/M_0$ as a function of the saturation pulse frequency, where $M_{sat}$ and $M_0$ are the bulk water magnetization measured after and without saturation, respectively (21). Traditionally, CEST effects are quantified using the asymmetric magnetization transfer ratio (MTR asym), which was first introduced by Zhou et al. (16). MTR asym measurements are calculated using:

$$\text{MTR}_{\text{asym}}(\Delta \omega) = \frac{M_2(\Delta \omega) - M_2(-\Delta \omega)}{M_2(-\Delta \omega)}$$

where $M_2(\Delta \omega)$ is the bulk water magnetization measured after saturation at the frequency $\Delta \omega$. However, some groups have found that nuclear Overhauser effects (NOEs) at frequency offsets $\Delta \omega$ between −2.0 and −4.0 ppm and asymmetric macromolecular magnetization transfer (MT) effects can significantly contaminate MTR asym measurements (22,23). Therefore, it is difficult to quantify CEST effects using the MTR asym technique (22). Recently, two novel techniques have been introduced to enable the quantification of amide and amine CEST effects in vivo, whilst avoiding field NOEs and asymmetric MT effects. Our group developed a CEST technique, called ‘amide and amine concentration-independent detection’ (AADCID), which uses the ratio of CEST effects of amide and amine ($\Delta \omega = 2.75$ ppm) protons to generate pH-dependent contrast independent of tissue macromolecule concentration and temperature (17). In addition, Jin et al. (23) have recently demonstrated a novel three-offset measurement technique to measure a parameter named ‘apparent amide proton transfer’ (APT*). Both AADCID and APT* techniques are sensitive to pH, and have been used in vivo to detect acidosis in cerebral ischemia models (17,23). In the current study, we compare the sensitivity of AADCID and APT* for the detection of LND-induced pH changes in tumors. The magnitude of single-dose LND-induced CEST contrast could help to predict tumor response to treatment.

**EXPERIMENTAL DETAILS**

**Subjects**

Nine mice were included in the current study: six NU/NU mice with U87 GBM brain tumors that were used to evaluate the effect of injection of 50 mg/kg ($n = 3$) and 100 mg/kg ($n = 3$) of LND; C57BL/6 male mice ($n = 3$) that were used to study the effects of LND on normal brain tissue. All animal procedures were performed according to a protocol that was consistent with guidelines established by the Canadian Council on Animal Care and was approved by the University of Western Ontario Animal Use Subcommittee.

**Lonidamine**

LND ($C_{13}H_{17}O_{2}C_{12}N_{2}O_{2}$) was purchased from Lancerix Chemicals (Shanghai, China). The drug (37.5 mg) was dissolved in 3 mL of pure dimethyl sulfoxide and vortexed until the solution was...
clear, LND was administered intraperitoneally at a dose of 50 or 100 mg/kg with a syringe pump (Harvard Apparatus, Holliston, MA, USA) over the course of 2 min.

Animal tumor preparation

GBM brain tumors were induced in 21–25-g NU/Nu mice (n = 6) using U87 GBM cells established from a human GBM (ATCC, Rockville, MD, USA), as described previously (24). Briefly, U87 GBM cells were grown in Dulbecco’s-modified Eagles medium supplemented with 10% fetal bovine serum (Wisent Inc., St-Jean-Baptiste, QC, Canada) at 37 °C in a humidified incubator with 5% CO2 and passaged twice a week. On the day of injection, U87 GBM cells were washed and dissociated with versene solution [phosphate-buffered saline (PBS) plus 0.5 mM ethylenediaminetetraacetic acid] and then washed twice with PBS, counted and re-suspended to a final concentration of 1 × 105 cells in 2 μL of PBS. Prior to injection, mice were anesthetized by inhalation of 4% isoflurane and maintained using 1.5% isoflurane. The mice were placed in a stereotactic head frame (Stoelting Instruments, Wood Dale, IL, USA). The scalp was swabbed with betadine and an incision was made in the scalp to expose the bregma. A 1-mm-diameter hole was drilled at coordinates measured from the bregma (1 mm anterior and 2 mm lateral). U87 GBM cells (2 mL) were injected at a rate of 0.5 μL/min, at a position 3 mm deep from the bregma, into the right frontal lobe using a Hamilton (Reno, NV, USA) syringe with a 27-gauge needle attached.

General mouse preparation for in vivo imaging

Anesthesia was induced using 4% isoflurane in oxygen and maintained with 1.5%–2.5% isoflurane in oxygen. Mice were secured on a custom-built, MRI-compatible stage and the head was secured using surgical tape to limit motion caused by respiration. Temperature was monitored with a rectal temperature probe, and respiration was monitored with a respiratory sensor pad connected to a pressure transducer that was placed on the thoracic region. The body temperature was maintained at 36.9–37.1 °C throughout imaging by blowing warm air over the animals using a model 1025 small-animal monitoring and gating system (SA Instruments Inc., Stony Brook, NY, USA). Following pre-injection imaging, the MRI-compatible stage was removed from the scanner without altering the animal position with respect to the stage. The mice were replaced in the scanner immediately after LND injection. Animals were sacrificed immediately after MRI.

In vivo imaging parameters

All animals were scanned using an Agilent (Santa Clara, CA, USA) 9.4-T, small-animal (31-cm horizontal bore) MRI scanner with an Agilent 30-mm millipede volume coil. The maximum gradient strength was 40 mT/m. Tumors were initially detected using a multi-slice standard anatomical T1-, T2- and diffusion-weighted MRI. On tumor detection, a slice of interest was selected with maximal tumor coverage and single-slice T1-, T2- and diffusion-weighted MR images were acquired. T1-weighted images were acquired using a standard fast spin-echo (FSE) pulse sequence (TR/TE = 500/10 ms; echo train length (ETL) = 4; time between excitation and the echo at the center of k-space (effective TE) = 10 ms; four averages; two pre-scans; field of view (FOV), 19.2 × 19.2 mm²; matrix size, 128 × 128; slice thickness, 2 mm; acquisition time, 1.1 min). T2-weighted images were acquired using an FSE pulse sequence (TR/TE = 3000/10 ms; ETL = 4; effective TE = 40 ms; one average; two pre-scans; FOV, 19.2 × 19.2 mm²; matrix size, 128 × 128; slice thickness, 2 mm; acquisition time, 1.7 min). Diffusion-weighted images were acquired using a multi-slice spin-echo pulse sequence (TR/TE = 1000/35 ms; b = 1100 s/mm² in the readout direction; four averages; FOV, 19.2 × 19.2 mm²; matrix size, 64 × 64; slice thickness, 2 mm; acquisition time, 8.5 min). CEST MR images were acquired for the slice of interest using an FSE pulse sequence (TR/TE = 7000/7 ms; ETL = 32; effective TE = 7 ms; three averages; two pre-scans; FOV, 19.2 × 19.2 mm²; matrix size, 64 × 64; slice thickness, 2 mm; acquisition time, 0.4 min; preceded by a 4-s-long, continuous-wave, RF saturation pulse with a B1 amplitude of 1.5 μT). z-Spectra were obtained by acquiring 57 CEST MR images, each with different saturation frequencies (in ppm), including −0.6 to 0.6 (Δ = 0.1), 1.6 to 4.5 (Δ = 0.1), 5.4 to 6.6 (Δ = 0.1) and 1000. In each animal, a B0 field map was generated for the image slice of interest immediately prior to each CEST experiment. A separate B0 map was acquired pre- and post-injection. B0 distribution maps were measured using the water saturation shift referencing (WASSR) technique (25). Specifically, a WASSR spectrum was acquired using an FSE sequence (TR/TE = 7000/7 ms; ETL = 32; effective TE = 7 ms; three averages; two pre-scans; FOV, 19.2 × 19.2 mm²; matrix size, 64 × 64; slice thickness, 2 mm; acquisition time, 0.4 min; 100-ms-long, continuous-wave, RF saturation pulse with a B1 amplitude of 0.2 μT; saturation frequencies, −0.5 to 0.5 ppm in 16 linearly spaced steps). The 16-point WASSR spectrum was interpolated to 401 points (resolution, 1 Hz) and fitted to a 10th-order polynomial. z-spectral data were frequency shifted to account for B0 variations. A B1 field map was created using an actual flip-angle imaging pulse sequence (TR = 20 ms; TE = 3.47 ms; number of echoes, 2; flip angle, 70°; one average; FOV, 19.2 × 19.2 mm²; matrix size, 64 × 64; acquisition time, 2.22 min) (26). For each pixel, B1 was calculated using $B1 = B1_{ref} * (actual \ flip \ angle, 70°)$ incorporating the flip-angle map and $B1_{ref} = 1.5 \ μT$. B1 variation was less than 5% throughout the brain and no B1 correction was necessary.

CEST data processing

All acquired CEST MR data were loaded into MATLAB (Mathworks, Natick, MA, USA) for analysis. Using custom MATLAB programs, B0 corrections were performed on a pixel-by-pixel basis and all z-spectra were smoothed using the ‘smooth’ algorithm from the MATLAB curve-fitting toolbox. Pre- and post-injection images were co-registered using custom MATLAB programs.

CEST and contrast calculations

Using B0-corrected and smoothed z-spectra, AACID values were measured on a voxel-by-voxel basis. AACID represents the ratio of CEST effects of amine protons resonating at 2.75 ppm and amide protons at 3.50 ppm, normalized by MT effects measured after saturation at 6.0 ppm, and is calculated using (17):

$$AACID = \frac{M_2(3.50 ppm) × (M_2(6.0 ppm) - M_2(2.75 ppm))}{M_2(2.75 ppm) × (M_2(6.0 ppm) - M_2(3.50 ppm))}$$

The bulk water magnetization following saturation at 6.0 ppm [i.e. $M_2(6.0 ppm)$] serves as a common reference point for both amine and amide CEST effects. As described previously in detail,
the 6.0-ppm normalization point may slightly reduce CEST sensitivity compared with asymmetry methods; however, it avoids contamination from NOEs that occur between −2.0 and −4.0 ppm and reduces the total number of MR images required (17). Furthermore, the common reference point at 6.0 ppm represents a good estimate of the endogenous MT contribution.

APT* measurements of CEST effects from the amide protons resonating at 3.50 ppm were also quantified using the simple three-offset measurement technique according to:

\[
APT^* = \frac{M_z(3.0 \text{ ppm}) - M_z(4.0 \text{ ppm}) - M_z(3.50 \text{ ppm})}{2.0} \quad \text{[3]}
\]

The contrast-to-noise ratio (CNR) was measured as described by Equation (4), where CESTBaseline represents the CEST parameters (AACID or APT*) measured at baseline and CESTLND represents the same parameters after LND treatment. The background noise (σBackground) was approximated as the standard deviation of the CEST parameters within normal tissue at baseline:

\[
\text{CNR} = \frac{\text{CEPTLND} - \text{CEPTBaseline}}{\sigma_{\text{Background}}} \quad \text{[4]}
\]

**Statistical analysis**

Mean AACID and APT* measurements were made from tumor and contralateral regions of interest (ROIs) for each mouse brain in quantitative CEST maps acquired before and after LND injection, as well as in LND-induced contrast maps. Differences in mean CEST values measured in tumor ROIs before and after LND injection were tested using Student’s paired t-test with the MATLAB statistics toolbox.

**RESULTS**

**CEST imaging of normal brain tissue following LND treatment**

A representative set of CEST images acquired from a healthy control animal (C57BL/6 male mouse) is presented in Figure 1. For each control mouse, CEST data were collected at baseline (i.e. immediately before LND injection) (Fig. 1a, d) and approximately 50 min after intraperitoneal bolus administration of 100 mg/kg LND (Fig. 1b, e). LND-induced contrast maps (Fig. 1c, f) show the spatial distribution of CNR calculated from the AACID (Fig. 1a, b) and APT* (Fig. 1d, e) distribution maps using Equation (4). Some small variations in LND-induced CNR are visible in the brain in both AACID and APT* contrast maps. Much of the variation in the contrast maps is spatially correlated to fine brain structures, such as ventricles, and may be attributed to motion between acquisitions. Some heterogeneity may also be attributed to variations in LND effects and delivery within normal brain tissue. Mean CEST values measured before and after injection of 100 mg/kg LND and LND-induced contrast are summarized for all control mice (n = 3) in Fig. 2. There were no significant differences between AACID whole-brain mean values acquired at baseline and after LND treatment. Similarly, no significant LND-induced changes were measured using APT*.

**Figure 1.** Representative set of chemical exchange saturation transfer (CEST) data from a healthy C57BL/6 mouse brain. CEST images acquired immediately before (a, d) and 50 min after (b, e) intraperitoneal injection of 100 mg/kg londamine (LND). Amine and amide concentration-independent detection (AACID) and apparent amide proton transfer (APT*) values were calculated from the same z-spectra. Contrast maps (c, f) were calculated using CEST maps acquired at baseline and after LND treatment employing Equation (4).

**CEST imaging of U87 GBM brain tissue following LND treatment**

Figure 3 presents a set of anatomical MR images for a representative NU/NU mouse, 14 days after U87 GBM cancer cell implantation. For all brain tumor mice, ROIs containing tumor (broken line) and normal contralateral tissue (full line) were drawn on the basis of anatomical changes observed in T2-weighted images, as shown in Figure 3a. The relatively low tumor contrast observed in the T2-weighted anatomical MR images (Fig. 3) is attributed to a combination of partial volume effects associated with the large (2-mm) slice thickness used and the expected similarity of the T2 relaxation time constants in the tumor and contralateral brain tissue (27,28). NU/NU mice with U87 GBM brain tumors were treated with 100 mg/kg (n = 3) or 50 mg/kg (n = 3) of LND. CEST MRI data are presented in Figures 3–7 for a representative NU/NU mouse with a U87 GBM brain tumor that was treated with 100 mg/kg LND. Figure 4 shows average baseline (blue) and post-injection (red) T2-Corrected z-spectra obtained using all pixels in the normal contralateral tissue-containing ROI (Fig. 4a) and tumor tissue-containing ROI (Fig. 4b). Baseline amide CEST peaks consistently showed larger amplitudes in tumors compared with contralateral tissue. Although amine protons resonating between 2.50 and 3.0 ppm did not generate clear CEST peaks, they also consistently generated larger CEST effects in tumor tissue at baseline. This effect is more easily visualized using the reference line superimposed on each baseline z-spectrum in Figure 4 with endpoints empirically set to M2/M0 values at 1.6 and 4.6 ppm. Furthermore, in Figure 4a, normal contralateral tissue z-spectra showed no significant change in amine CEST effect at 2.0 and 2.75 ppm, or amide CEST effect at 3.50 ppm (i.e. both spectra overlap), following LND injection. A small vertical offset is visible between spectra at saturation frequencies greater than ~2.50 ppm which may be caused by a minor change in macromolecular MT effects. Conversely, more distinct changes were observed in the z-spectrum of the tumor ROI after LND injection (Fig. 4b). Figure 4b shows a marked decrease in the amide CEST effect between 3.0 and 4.0 ppm following LND injection. This change strongly suggests LND-induced tumor acidification as amide CEST effects decrease in tissue on acidosis. In tumor ROIs, CEST spectra demonstrated a very small change in amine CEST effect at 2.0 ppm and no change in amine CEST effect at 2.75 ppm, as expected from previous work (17). Notably, the minor vertical offset noted above in normal tissue spectra was not observed in tumor spectra.
Figure 2. Summary of chemical exchange saturation transfer (CEST) and contrast values from healthy C57BL/6 mice (n = 3). Mean amine and amide concentration-independent detection (AACID) (a) and apparent amide proton transfer (APT*) (b) values acquired at baseline and approximately 50 min after 100 mg/kg lonidamine (LND) treatment. No significant differences were observed before and after LND treatment in both the AACID and APT* values. As a result, the average LND-induced contrast was equal to background noise. CNR, contrast-to-noise ratio.

Figure 3. Standard anatomical MRI of U87 glioblastoma multiforme (GBM) brain tumor before injection of lonidamine. (a) T2-weighted image with tumor (broken line) and contralateral (full line) regions of interest (ROIs) drawn. T1-weighted (b) and diffusion-weighted (c) images were also acquired using the identical slice of interest.

Figure 4. Chemical exchange saturation transfer (CEST) z-spectra collected at baseline and approximately 50 min after the administration of lonidamine (LND). Average B0-corrected z-spectra calculated from normal contralateral tissue-containing region of interest (ROI) (a) and tumor tissue-containing ROI (b) with error bars equal to one standard deviation. A reference line (broken gray line) was empirically superimposed onto the baseline z-spectra to aid the visualization of differences between the z-spectra in contralateral and tumor tissue. These zoomed z-spectra highlight the significant changes in the 1.6–4.5-ppm frequency range.

Figure 5 shows quantitative CEST distribution maps measured on a voxel-by-voxel basis using CEST data measured before (Fig. 5a, d) and after (Fig. 5b, e) injection of 100 mg/kg LND. At baseline, tumor regions consistently presented lower AACID and elevated APT* measurements (Fig. 5a, d, respectively) compared with normal tissue. The average tumor CNR values (n = 6) for each CEST parameter (CEST parameter: tumor mean CNR ± standard error), calculated using CEST contrast maps, were as follows: AACID, 4.99 ± 1.11; APT*, 5.12 ± 0.82. Approximately 50 min after LND injection, a marked increase in AACID and a reduction in APT* values were observed in tumor regions only (Fig. 5b, e, respectively). Conversely, tissue relaxation time constants and/or tissue water content appeared to be unaffected by LND treatment, because no difference was observed between T1-, T2- and diffusion-weighted images acquired before and after injection. Contrast maps (e.g. Fig. 5c, f) were used to locate and quantify changes in AACID and APT* caused by 100 mg/kg LND treatment. A marked increase in spatial heterogeneity was found in LND-induced contrast maps generated using both APT* and AACID within tumor regions, which was not observed in the pre-injection CEST maps. Figure 6 summarizes CEST data from all brain tumor animals treated with 100 mg/kg LND (n = 3),
calculated using each pixel in tumor and contralateral tissue-containing ROIs from baseline and post-injection images, as well as contrast maps. In tumor ROIs, AACID values were consistently and significantly higher after LND injection relative to baseline ($p < 0.05$ by $t$-test, $n = 3$). Similarly, APT* values showed a trend towards lower values in tumor ROIs after LND injection relative to baseline ($p = 0.078$ by $t$-test, $n = 3$). The LND-induced signal change was significantly higher in tumor regions relative to normal tissue using either CEST measure (Fig. 6). Therefore, both AACID and APT* contrast maps produced high tumor contrast and could potentially be used to detect tumor-specific effects of LND injection. The LND-induced CNRs generated using AACID and APT* values were not significantly different. At approximately 50 min after 100 mg/kg LND treatment, the average CNR values ($n = 3$) for each CEST parameter (CEST parameter: tumor mean CNR ± standard error; contralateral mean CNR ± standard error), calculated using CEST contrast maps, were as follows: AACID, 4.54 ± 1.79; 1.41 ± 1.06; APT*, 4.82 ± 2.51; 1.81 ± 0.51.

To investigate the dose dependence of LND-induced AACID and APT* contrast, a set of NU/NU U87 GBM brain tumor mice ($n = 3$) was treated with 50 mg/kg LND. The LND-induced contrast in tumors generated at 50 mg/kg was consistently lower than that in animals treated with 100 mg/kg LND for both AACID and APT*. No significant difference was observed between AACID and APT* LND-induced contrast maps in animals treated with 50 mg/kg LND. Figure 7 summarizes the dose dependence of the mean contrast values generated using each pixel in tumor ROIs in AACID and APT* maps following LND treatment at doses of 50 mg/kg ($n = 3$) and 100 mg/kg ($n = 3$). At approximately 50 min after 100 mg/kg LND injection, the mean AACID and APT* values were 4.54 ± 1.79 and 4.82 ± 2.51, respectively, in tumor ROIs, and 1.41 ± 1.06 and 1.81 ± 0.51, respectively, in contralateral ROIs (error bars = ± standard error). No significant difference was observed between AACID and APT* LND-induced contrast maps in animals treated with either dose.
50 min after 50 mg/kg LND treatment, the average CNR values \( (n = 3) \) for each CEST parameter (CEST parameter: tumor mean CNR ± standard error; contralateral mean CNR ± standard error), calculated using CEST contrast maps, were as follows: AADCID, 3.24 ± 0.66; 1.21 ± 1.34; APT*, 3.96 ± 1.94; 1.82 ± 1.25.

Histological analysis of U87 GBM brain tumors following LND treatment

Following MRI experiments, animals were killed and two brains were excised for histology. Brain sections (5 μm thick) corresponding to the selected slice of interest were stained for morphological identification using hematoxylin and eosin (H&E). For the representative animal presented in Figures 3–5, H&E-stained brain sections confirmed that regions of high LND-induced CNR within CEST contrast maps corresponded to U87 GBM tumor regions, as shown in Figure 8. Two regions of heterogeneous H&E stain uptake that were observed within the tumor (labeled with boxes b and c in Fig. 8a) are attributed to vasculature and/or inflammatory cells. Interestingly, regions b and c appear to correspond spatially to regions of heterogeneity observed in the LND-induced CNR maps (Fig. 5c, f). However, robust registration of the LND-induced CNR map with the brain section is required to conclude that the regions are correlated. Some signal variation could also be attributed to motion, as observed in the LND-induced CNR maps (Fig. 5c, f). However, robust registration of the LND-induced CNR map with the brain section is required to conclude that the regions are correlated. Some signal variation could also be attributed to motion, as observed in the AACID and APT* contrast maps obtained in healthy mice (Fig. 1).

DISCUSSION

The goal of this study was to determine whether CEST MRI contrast changes in response to single-dose LND treatment could be used to detect treatment response in orthotopic GBM tumor. LND is an anticancer agent that alters metabolism and pH\( _i \). The results indicate that both the AACID and APT* CEST parameters studied are sensitive to LND-induced pH\( _i \) changes in tumors within 1 h of treatment, and that the changes observed are roughly four-fold higher in tumors than in surrounding brain tissue. Both AACID and APT* provided similar dose-dependent CNR.

The use of CEST contrast has many potential clinical applications. Zhou et al. (16) were the first to show that exchanging protons associated with endogenous mobile proteins and peptides could be used to detect changes in pH\( _i \), employing amide CEST contrast. Endogenous amide and amine CEST contrast has since been used to detect decreased pH\( _i \) in the ischemic penumbra after cerebral ischemia (16,23). Amide CEST contrast in tumor regions has also been used to detect tumors (29). Recently, Zhou et al. (5) have discovered that differences in amide CEST contrast can be used to differentiate recurrent glioma from radiation necrotic tissue. Some groups have also found that amide and amine CEST effects are sensitive to necrotic regions within tumors (18,30). In addition, as already mentioned, Sagiyama et al. (3) discovered that amide proton CEST effects could detect temozolomide treatment effects 1 week after treatment.

The amide CEST peak amplitude is consistently larger in tumors than in normal tissue. Large amide CEST peak amplitudes in tumors are typically attributed to several characteristics of tumor tissue, including longer \( T_1 \) and \( T_2 \) relaxation time constants, a more dilute macromolecular pool with lower MT effects, slightly alkaline pH\( _i \), and increased mobile cytosolic protein concentration (15,18). Amide protons originate from the amide groups in the backbone of endogenous proteins and peptides. Similarly, relatively slow-exchanging amine protons resonating between 2.0 and 3.0 ppm originate from lysine amino acids in proteins and peptides.

In the current study, LND treatment caused the amide proton CEST peak to decrease significantly in tumor regions only. Base-catalyzed amide proton exchange rates decrease exponentially with a decrease in pH\( _i \), and therefore local acidosis results in reduced CEST effects \textit{in vivo} (16,17,23). Amine protons resonating at 2.0 and 2.75 ppm also have base-catalyzed exchange; however, LND treatment induced only small changes in CEST at 2.0 ppm and no change at 2.75 ppm. The difference in pH sensitivity is attributed to the unique ionization constants (pK\(_a\)) of the different proton pools (20). Recently, our group showed that amine protons associated with proteins and peptides that resonate at 2.75 ppm exchange in the slow-to-intermediate regime at neutral pH \textit{in vitro} (17). However, \textit{in vivo}, consistent with the observations in the current study, we were previously unable to detect pH\( _i \)-dependent changes in CEST effects at 2.75 ppm in acidic tissue regions after stroke (17). The insensitivity of amine CEST effect to changes in pH\( _i \) could be predicted on the basis of the relatively low power saturation scheme used (i.e. 4 s, 1.5 μT continuous wave saturation) that is not optimally tuned to faster

Figure 8. Hematoxylin and eosin (H&E)-stained brain section from representative U87 glioblastoma multiforme (GBM) brain tumor model. (a) Brain section corresponding to the slice of interest used for the chemical exchange saturation transfer (CEST) MR images in Figure 3, with two regions of interest that display stain heterogeneity attributed to vasculature and/or inflammatory cells, labeled ‘b’ and ‘c’. (b, c) Magnified regions of interest ‘b’ and ‘c’, respectively.
show that tumor pH, decreased by 0.45 pH units after injection of 100 mg/kg LND and by approximately 0.25 pH units after injection of 50 mg/kg LND. Assuming that LND injections in the current study generated similar changes in tumor pH, per LND dose, our results indicate that both APT* and AACID parameters are sufficiently pH sensitive to detect pH changes of ~0.25 pH units, as predicted in previous work (16,17).

The key finding of this study is that amide proton-mediated CEST effects decreased significantly in GBM tumor regions immediately after LND treatment. Consequently, post-injection quantitative APT* and AACID distribution maps showed differences relative to baseline maps in tumor regions only. Tumor-specific drug effects were evident approximately 1 h after dosing. LND-induced contrast maps offered high tumor contrast, consistently enabling accurate tumor detection and boundary delineation. The primary results presented here suggest that the technique of CEST imaging before and after application of a physiological stress could offer a novel method for tumor detection. To be clinically relevant, this technique must be operationalized using a physiological stress that is well tolerated by the patient, and must offer improved tumor contrast compared with baseline images. In the current study, the LND/dimethyl sulfoxide injection was not well tolerated by mice. However, in some mice, LND-induced tumor contrast was higher than the contrast between contralateral and tumor tissue in the baseline CEST maps. Further work is required to evaluate the tolerability of alternative physiological stressors that could further improve tumor contrast. No significant differences were found between APT* and AACID parameters in terms of LND-induced tumor contrast. The heterogeneity observed in tumor regions of the APT* and AACID contrast maps correlated with morphological variations observed in H&E-stained histology images (Fig. 8). Morphological variations may be attributed to vasculature and/or inflammatory cells, which should be further investigated; however, this was beyond the scope of the current work. Importantly, H&E-stained histology slices consistently demonstrated minimal necrosis throughout the solid tumors, as shown in Figure 8. However, as necrotic tissue is common in humans with GBM (36), LND-induced changes in CEST effects should also be measured in necrotic tissue. If necrotic tissue is unresponsive to LND, the use of a physiological stress combined with CEST imaging may offer a new approach to differentiate necrotic tissue from tumor and normal tissue.

The single-dose LND CEST method could be translated to lower field strength clinical MRI scanners, which are routinely used to produce APT contrast in humans (37–39). Several clinical trials investigating cancer treatment using LND in the USA, Canada and across Europe have reached phase II and III status (40,41); however, high liver toxicity at 3 months of dosing led to a hold on all clinical trials in the USA in 2006 (42). Recently, cancer-targeted LND-containing liposomes have been developed and have been shown to improve adjunct chemotherapy in a small-animal tumor model (42). Importantly, the dose of LND used in the current study was 100 mg/kg, which is considerably higher than that used clinically in the treatment of cancer in adult humans, where the highest reported total single-day dose was 900 mg. The main side-effects associated with LND treatment are typically mild and include myalgia, asthenia and testicular pain (41,43). The dose in the current study was chosen to replicate previous studies in the MR literature (9–11,14). Similar studies (9,10) have reported high toxicity when using high LND doses (i.e. >50 mg/kg); however, Nath et al. (10) and Ben-Yoseph...
et al. (11) found mortality to be completely avoided when dissolving LND in a Tris/glycine buffer. In the current study, the authors were unable to completely dissolve LND unless using pure dimethyl sulfoxide as a solvent.

The small sample size represents a limitation of the present study. In addition, absolute pH was not measured because the calibration between AACID CEST and absolute pH has not been established in U87 GBM. Finally, this study was not designed to evaluate the predictive value of LND-induced pH-dependent changes in CEST contrast with a therapeutic outcome. Future longitudinal studies will be designed to follow animals following treatment.

Further work is required to investigate whether other clinically approved therapeutic agents can alter tumor pH after single-dose administration, and whether these changes are detectable by CEST MRI. In addition, future investigations must determine whether acute pharmacologically induced CEST contrast predicts treatment outcomes. Finally, tumor-selective LND effects were so clear that safer pharmacological stressors should be investigated in combination with CEST MRI for applications in cancer detection, differentiation and grading.

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