Supplementary Information

A General MRI-CEST Ratiometric Approach for pH Imaging: Demonstration of *In Vivo* pH Mapping with iobitridol

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Experimental Procedures

Phantom studies

Two types of phantoms were used to calibrate iobitridol pH dependence and to assess concentration independence. pH calibration curves were measured on 30 mM iobitridol (Guerbet, Roissy CdG Cedex, France) phosphate buffered solution with pH titrated to 5.5, 6, 6.3, 6.7, 7, 7.4 and 7.9 (EuTech pH Meter, Singapore). Concentration independence measurement was assessed by preparing iobitridol solution at 5 different concentrations: 10, 20, 30, 40 and 50 mM titrated at two different pH values of 6.6 and 7.2. Prepared solutions were transferred into eppendorf tubes and inserted into a phantom container filled with water.

In vitro CEST MRI

All images were acquired with a 7 T Bruker Avance 300 MHz MRI system equipped with a 30 mm quadrature RF coil. For in vitro experiments, a modified single-shot rapid acquisition with refocusing echoes (RARE) sequence (TR = 8.0 sec, effective TE = 37ms, RARE factor = 64, centric encoding, slice thickness = 4 mm, FOV = 30 mm, matrix size = 64x64, NA = 4) including a magnetization transfer module (continuous wave (CW) irradiation, B_1 = 1.5, 3, and 6 μT for 5 sec) was used to acquired CEST images with 201 frequency

offsets evenly distributed from -10 ppm to 10 ppm (step size = 0.1 ppm) around the water resonance (0 ppm). Temperature was set to 310 K and maintained throughout the experiments using a warm-air feedback system; temperature calibration was performed by looking to the chemical shift separation of a neat methanol solution.

Animal Model

TSA is an aggressive and poorly immunogenic cell line first established from the in vivo transplant of a moderately differentiated mammary adenocarcinoma that arose spontaneously in a 20-month-old multiparous BALB/c mouse (provided by Dr. Federica Cavallo, Molecular Biotechnology Center, University of Torino, Torino – Italy). TSA cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2mM glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin and preserved in a humified incubator at 37°C with 5% CO_2. All animal-related procedures were approved by the Institutional Animal Care and Use Committee at University of Torino. All animals were maintained under specific pathogen-free conditions inside the animal facility and received standard rodent chow and had free access to tap water. BALB/c mice (20-24 g, 8-10 weeks old) were anesthetized by injecting intra-muscle a mixture of tiletamine/zolazepam (Zoletil 100; Virbac, Milan, Italy) 20 mg/kg and xylazine (Rompun; Bayer, Milan, Italy) 5 mg/kg. One mouse was injected with $2.5x10^5$ TSA cells in both the right and left flanks to allow tumors to grow to 4-6 mm diameter after 14 days. Animal position was secured using an MRI-compatible animal holder, and respiratory rate was monitored throughout in vivo MRI (SAII Instruments, Stony Brook, NY - USA).

In vivo CEST-MRI

All images were acquired with a 7 T Bruker Avance 300 MHz MRI system equipped with a 30 mm quadrature RF coil. A single T_2-weighted axial slice crossing the centers of the kidneys or of the tumors was acquired with TR = 4 sec, TE = 3.7 ms, NA =1, slice thickness = 1.5 mm, FOV = 30x30 mm, matrix size = 64x64, which yielded an in-plane resolution of 470 µm. CEST images were acquired with continuous wave (CW) RF irradiation (1.5 and 6 µT for 5 sec) by using a single-shot RARE sequence (TR = 6.0 sec, effective TE = 37ms, RARE factor = 64, centric encoding, slice thickness = 1.5 mm, FOV = 30 mm, matrix size = 64x64, NA = 1) with 39 frequency offsets unevenly distributed from -10 to 10 ppm relative to the water resonance, with the acquisition time for each Z-spectrum being 4 min 20s. The contrast agent was injected intravenously into the tail vein at a dose of 1.5 g I/kg b.w. for kidneys pH mapping and at a dose of 4 g I/kg b.w. for tumor pH mapping.

In vivo pH validation

To validate pH values obtained with the proposed ratiometric procedure, a comparison with the conventional ratiometric approach was performed by using iopamidol. Four healthy balb/c mice (10-12 weeks old, mean weight 20-24 g) were imaged following intravenously iobitridol injection (dose 1.5 g I / kg b.w.) and the day after by intravenous injection of iopamidol (dose 1.5 g I / kg b.w.). CEST images were acquired as described before for iobitridol, for iopamidol the same settings were used, but the number of frequency offsets that was increased to 43, to cover the frequency offsets of 4.2 ppm and 5.5 ppm. pH

maps were obtained, for iobitridol, as described below, while for iopamidol exploiting the pH calibration curve described in prior paper (Magn Res Med 2011, 65, 202). Differences in mean pH between the two methods were tested using the Student's t-test. Pearson correlation analysis was performed between each mean pH measured by the two methods. The statistical analysis was carried out using the program for Graphpad PRISM, version 5.01. A p-value of less than 0.05 was considered statistically significant.

CEST MRI processing

All data analysis was performed using custom-written scripts in Matlab (Mathworks, Natick, MA, USA).

All Z-spectra were interpolated, on a voxel-by-voxel basis, by smoothing splines, to identify the right position of the bulk water signal, for B_0 inhomonogeneity correction.[1] The interpolated Z-spectrum was shifted so that the bulk water resonance corresponds to the zero frequency and CEST contrast was quantified at a specific offset of interest (i.e. Δω= +5.6 ppm) using the asymmetry analysis:

$$ST = \frac{S^{-\Delta\omega} - S^{\Delta\omega}}{S_0}$$

Where $S^{\pm\Delta\omega}$ is the water signal intensity in the presence of a saturation pulse at offset ±Δω and S_0 is water signal intensity in the absence of a saturation pulse. pH calibration curves were obtained by using Eq. 1 as reported in the main text:

$$RPM = \frac{\left[(1-ST)/ST\right]_{RF1}}{\left[(1-ST)/ST\right]_{RF2}} \qquad (1)$$

Where RPM is the ratiometric value obtained upon ratioing the ST contrasts at two different RF irradiation power levels of RF_1 and RF_2.

For in vivo images, difference contrast maps (ΔST) were calculated by subtracting the ST contrast after iobitridol injection from the ST contrast before the injection on a per voxel basis in order to reduce the confounding effect of the endogenous contributions. STmaps were calculated for both the irradiation power levels of 1.5 μT and of 6 μT, by subtracting the corresponding pre-contrast ST maps. A threshold value of 1 and 2% was set, based on the ΔST variations between multiple pre-contrast ST maps (ca. 0.8% at 1.5 μT and 1.7% at 6 μT, respectively) to discriminate between enhancing and not-enhancing pixels. Ratiometric values were calculated by ratioing the difference contrast maps at the two different RF irradiation power levels of 1.5 μT and of 6 μT. Only those voxels showing an increase of ST contrast higher than 1% and 2%, for in comparison to pre-contrast ST map were included for the following pH calculations. pH maps were obtained by back-calculating the pH values from the obtained ratiometric values on a voxel-by-voxel basis according to the corresponding pH calibration curve of Figure 2b.

Extravasation experiments

A comparison of the amount of extravasation between iobitridol and ProHance (Bracco Imaging, Milan, Italy), two contrast agents sharing comparable molecular weights (835 g/mol and 559 g/mol for Iopamidol and ProHance, respectively) was assessed by sequential injection in a TSA tumor bearing mouse. After the

scout image acquisition, T_{2w} anatomical images were acquired with a Fast Spin Echo sequence and the same geometry was used for subsequent CEST and T_{1w} experiments. CEST acquisition was performed as described above for tumor pH mapping. Baseline and post-contrast injection tumor T_{1w} images were acquired by using an axial T_{1w} 2D spoiled Gradient Echo sequence with the following parameters: TR/TE = 10/4.1 ms, flip angle = 65°, number of slices = 5, slice thickness = 1.5 mm, FOV = 30 mm, matrix = 128x128. ProHance (Bracco Imaging, Italy) was injected into the tail vein through a 27-gauge needle at a dose of 0.1 mmol Gd/kg. Contrast-enhanced images were calculated for iobitridol, by calculating contrast difference map at 5.6 ppm 10 min post injection, while for Prohance the enhancement maps was calculated as the percentage of enhancement between pre and post-contrast T_{1w} images at the same time post-injection.

In vivo concentration independence

The proposed new approach requires the acquisition of two whole Z-spectra instead of only one as with the conventional ratiometric approach; therefore, contrast agent concentration changes between two Z-spectra may affect pH determination. To validate the *in vivo* concentration independence, an experiment was performed by alternating the acquisition at two RF power level of 1.5 and 6 µT post-injection (6 - 1.5 – 6 - 1.5 – 6) in a TSA-tumor bearing mouse following iobitridol injection (dose 4 g I / kg b.w.). The repeated Z-spectra were analyzed with the proposed ratiometric approach by picking the first two images (6/1.5 µT), the second and the third ones (1.5/6 µT), the third and the fourth ones (6/1.5 µT) and the fourth and the fifth images (1.5/6 µT) for calculating the corresponding pH maps by taking the ratio of the difference contrast maps.

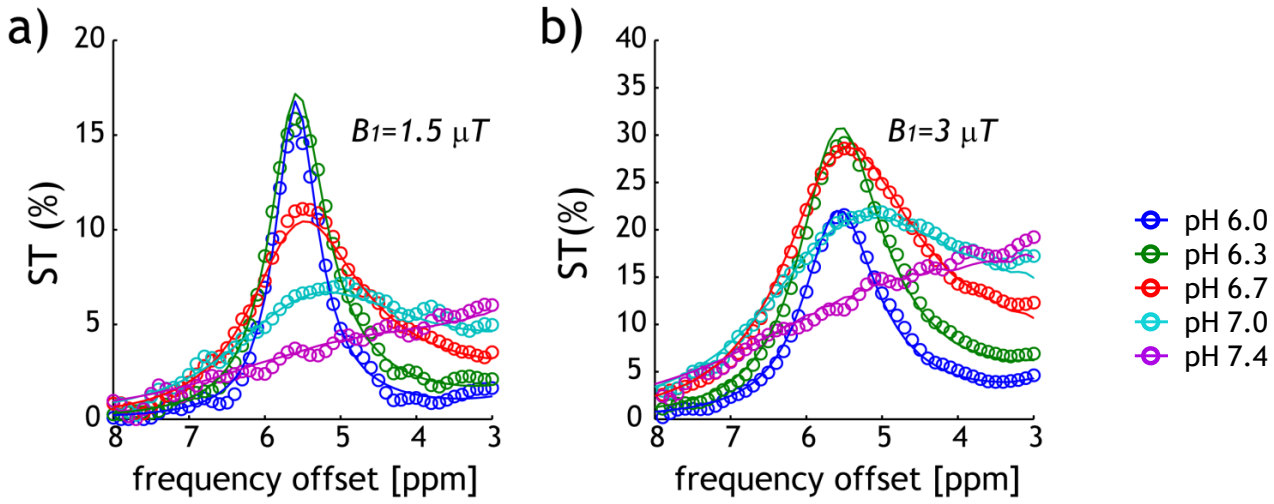

Figure S1. Numerical solution of pH-dependent chemical exchange properties of iobitridol (B_0 = 7T, 310K). Asymmetry plots in the range 3 to 8 ppm were numerical solved for B_1 = 1.5 µT (a) and 3 µT (b).

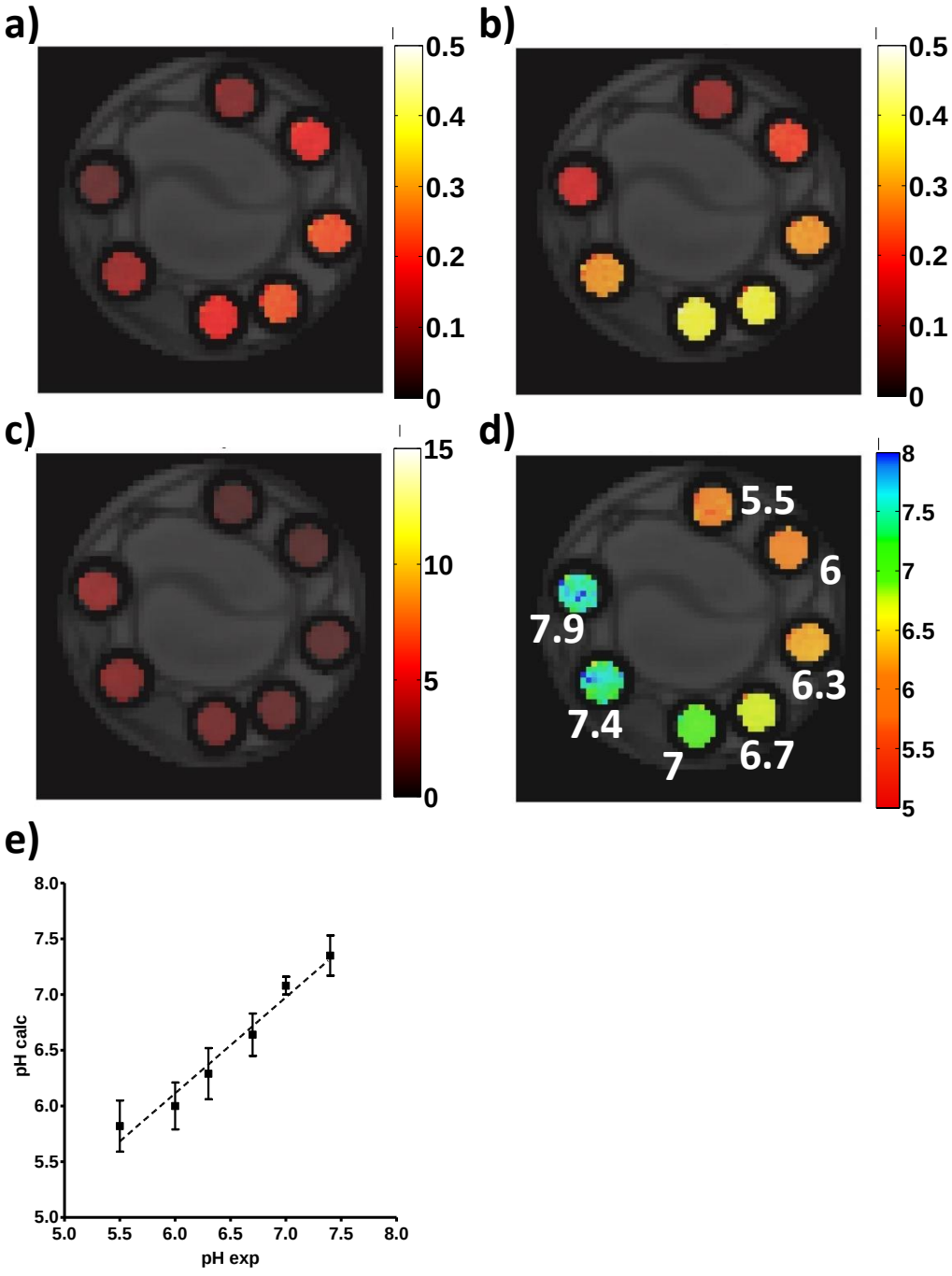

Figure S2. CEST-MR images of 30 mM iobitridol solution titrated at different pH values (5.5 - 6.0 - 6.3 - 6.7 - 7.0 - 7.4 - 7.9); ST images obtained upon irradiation with RF saturation levels of 3 µT (a) and 6 µT (b); (c) ratiometric RPM map calculated by using equation [1] from the ratio of the corresponding ST images S2a and S2b; (d) pH map calculated from the ratiometric map and the calibration curve of Figure 2b; (e) pH calculated vs. pH titrated for 30 mM iobitridol phantoms, R^2=0.97 (B_0 = 7T, 310K).

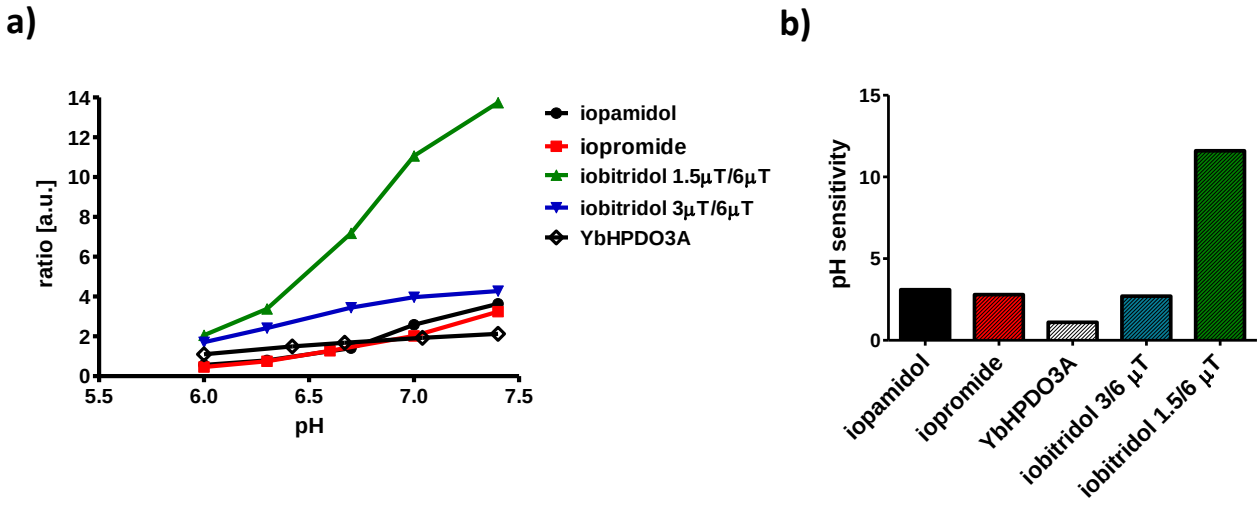

Figure S3. Comparison of pH MRI sensitivity of pH-responsive CEST-MRI contrast agents. (a) pH responsiveness of iopamidol (black circles), iopromide (red squares) and YbHPDO3A (open diamonds) were calculated by exploiting the conventional ratiometric procedure following the ratioing of the ST contrast for the two mobile proton pools irradiated with the same B_1 power level (3 µT for 5 sec for Iopamidol and Iopromide, and 24 µT for 1.5 sec for YbHPDO3A, respectively). pH responsiveness of iobitridol was assessed by exploiting the proposed ratiometric pH MRI method following the ratioing of the ST contrast generated by two different B_1 saturation levels (3 over 6 µT for 5 sec, blue down-pointing triangle and 1.5 over 6 µT for 5 sec, green up-pointing triangles, respectively). (b) pH sensitivity (ΔRpH) measured as the ratiometric difference between pH levels of 7.4 and 6.(All results were measured on 30 mM solutions in phosphate buffer, B_0 = 7T, 310K).

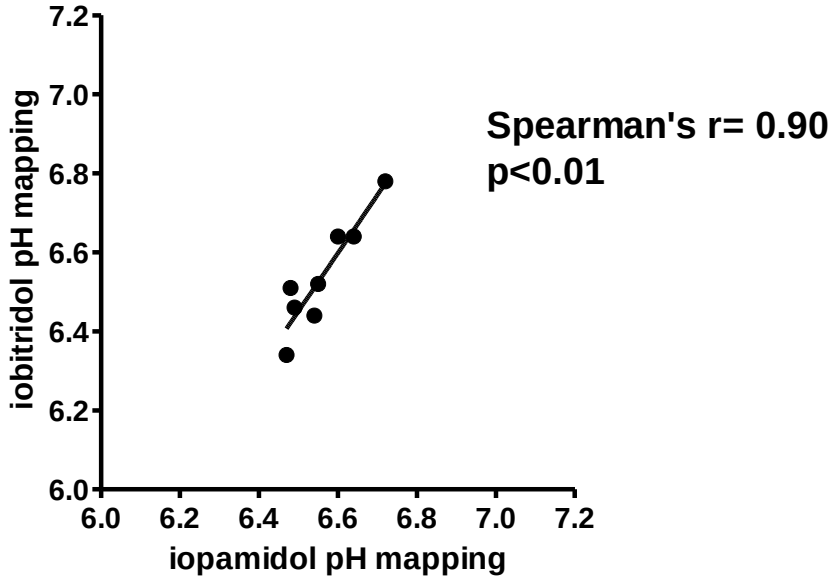

Figure S4. Correlation between mean pH measured by conventional ratiometric approach using iopamidol and the proposed new ratiometric method using iobitridol.

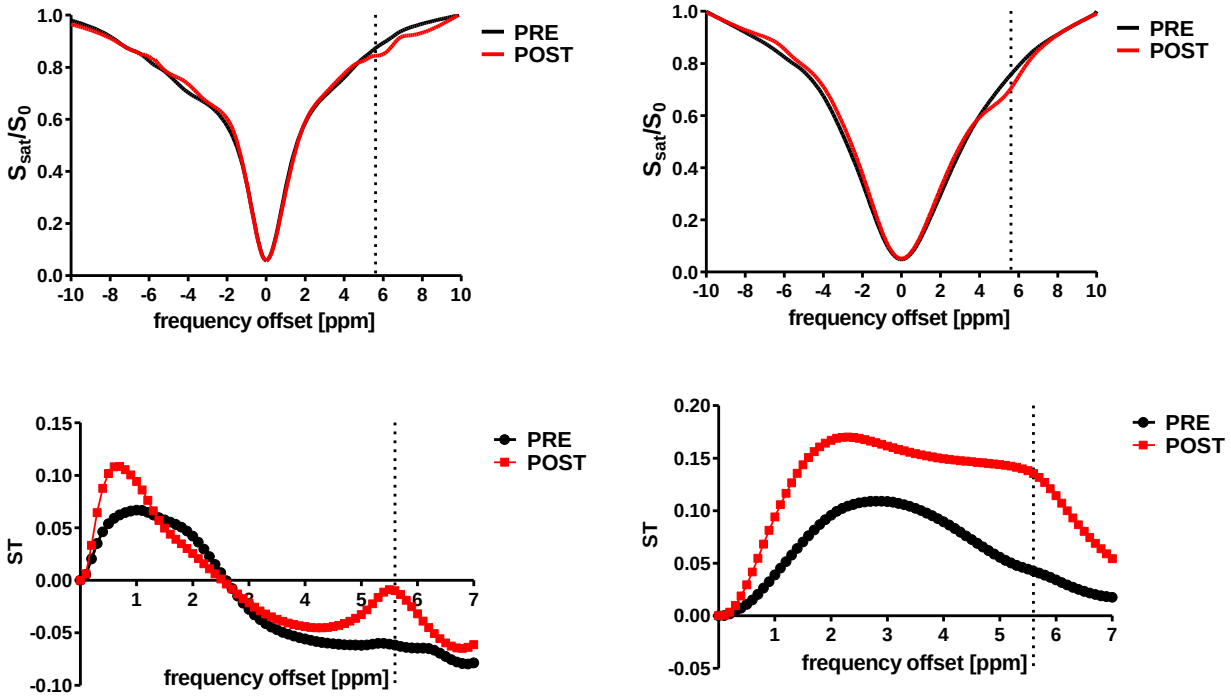

Figure S5. Representative Z-spectra and ST curves from ROI including the kidneys of Figure 5 before (black) and after (red) iobitridol injection at a dose of 1.5 g Iodine / kg b.w. following a saturation pulse of 1.5 µT (left, (a) Z-spectra and (c) ST) and a saturation pulse of 6 µT (right, (b) Z-spectra and (d) ST curves).

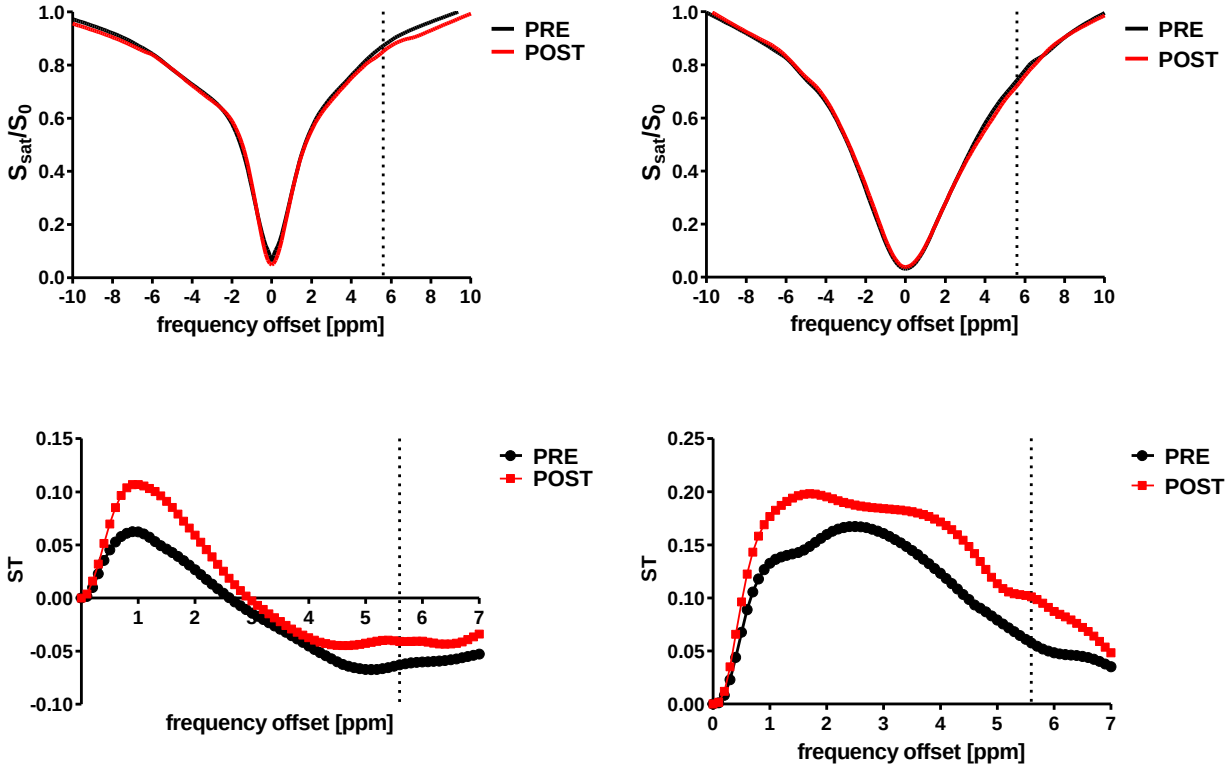

Figure S6. Representative Z-spectra and ST curves from ROI including the tumor of Figure 6 before (black) and after (red) iobitridol injection at a dose of 4 g Iodine / kg b.w. following a saturation pulse of 1.5 µT (left, (a) Z-spectra and (c) ST) and a saturation pulse of 6 µT (right, (b) Z-spectra and (d) ST curves).

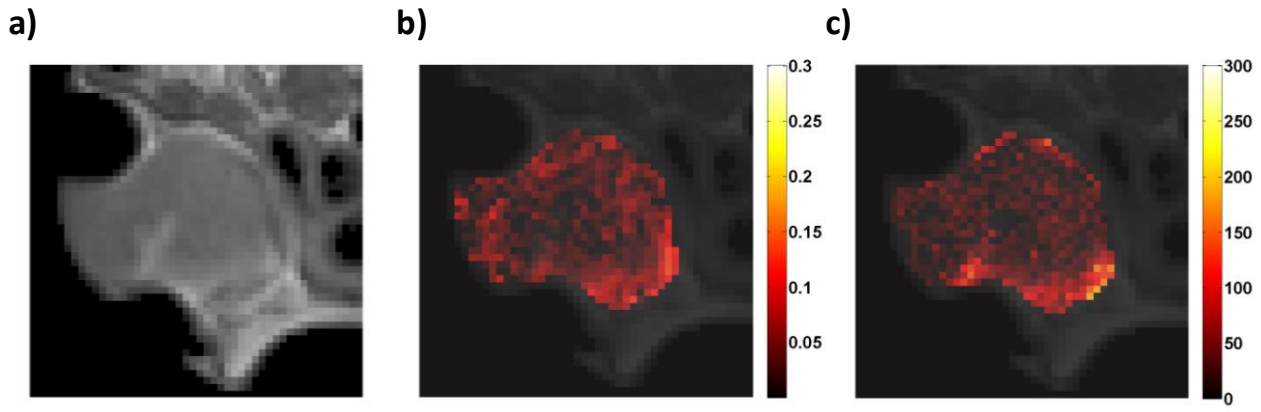

Figure S7. MRI contrast map in a TSA tumor bearing mouse following sequential injection of iobitridol and of ProHance. (a) T_2-weighted anatomical image zoomed to the tumor region; (b) CEST difference map at 5.6 ppm (STpost – STpre iobitridol injection, dose = 4 g Iodine / kg b.w.) ; (c) Contrast Enhancement map ((SIpost-SIpre)/SIpre*100) following ProHance injection (dose = 0.1 mmol Gd / kg b.w.)

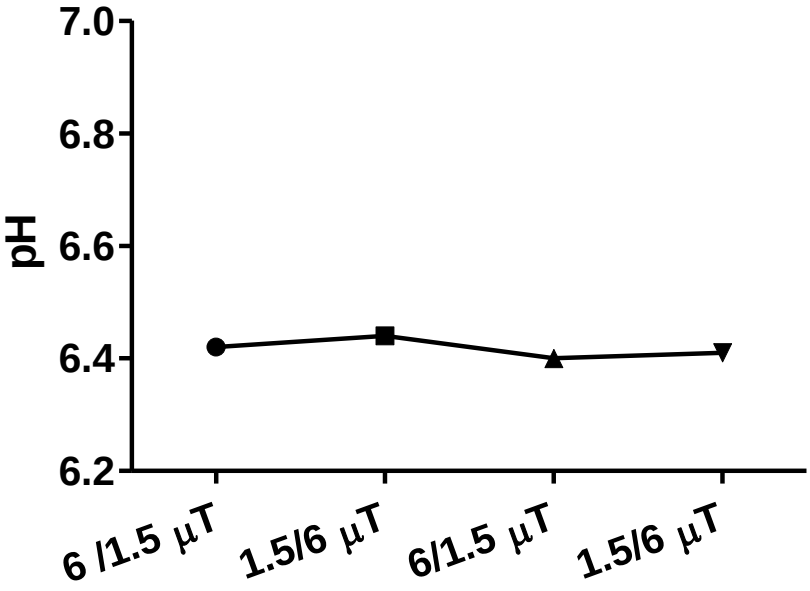

Figure S8. Tumor pH measurements obtained by alternating Z-spectra acquisition at 6 µT and at 1.5 µT power levels following iobitridol i.v. injection (dose 4 g I / kg b.w.) in a TSA tumor-bearing mouse. Corresponding pH values were calculated by picking the first two Z-spectra (6/1.5 µT), the second and the third Z-spectra (1.5/6 µT), the third and the fourth Z-spectra (6/1.5 µT) and the fourth and the fifth Z-spectra (1.5/6 µT). The results showed that small change in iobitridol concentration due to washout does not substantially affect pH determination.

References

(1) Stancanello, J.; Terreno, E.; Castelli, D. D.; Cabella, C.; Uggeri, F.; Aime, S. *Contrast Media Mol Imaging* **2008**, *3*, 136-49.