Supplemental Information

Zhou R et al "Glutamate-weighted Chemical Exchange Saturation Transfer (GluCEST) Magnetic Resonance Imaging Detects Glutaminase Inhibition in a Mouse Model of Triple Negative Breast Cancer"

Supplemental Methods

Tumor inoculation, treatment, imaging and tissue collection schedule

All animal studies were approved by the animal care and usage committee (IACUC) of the University of Pennsylvania. Xenografts from HCC1806 cells were established in NCR-athymic nu/nu mice (female, 7 weeks old, Charles River) as we described previously (1). Briefly, 1 million HCC1806 cells in PBS were implanted subcutaneously in the flank of the mouse.

We treated tumor-bearing mice with a highly selective, potent and reversible inhibitor of *GLS*, CB-839, which was dissolved in the vehicle solution (VEH) of 25% (w/v) hydroxypropyl-β-cyclodextrin in 10 mmol/L citrate (pH 2), and was administered via oral gavage (200 mg/kg twice daily); control mice received the same volume (0.25 mL) of VEH. A total of 4 doses (over 2 days) of CB-839 or VEH were administered. This short treatment course did not induce substantial changes in tumor volume while ensuring sufficient tumor exposure to the drug.

The inhibitory effect of CB-839 on GLS is concentration-dependent and reversible, meanwhile the plasma peak concentration of CB839 is reached ~ 4 h post oral administration (2). Therefore we set up two cohorts, one for in vivo GluCEST MRI and the other for collection of tumor tissues. GluCEST imaging and tissue collection were performed at ~4 h after the last gavage to ensure peak plasma concentration of the drug at the time of imaging and tissue collection.

In vivo measurement of tumor intracellular pH by ^{31}P MRS

In vivo tumor-localized ^{31}P MRS was performed using the image-selected-in-vivo-spectroscopy and the intracellular pH was estimated by the chemical shift of inorganic phosphate resonance using Henderson-Hasselbalch relationship as we reported (3).

^{1}H MR spectroscopy quantification of glutamate from tumor and cell extracts

Glutamate (Glu) and other water-soluble metabolites concentration in the cells and tumors was estimated by ^{1}H magnetic resonance spectroscopy (MRS) of aqueous extraction of cell pellet or tumor. After HCC1806 cells were incubated with 1 µM CB839 or 0.05% DMSO in culture media for 24 h, cell pellet (from ~10^7 cells) was collected by centrifuge (1000 g for 10 min). Methanol:water (1:1 v:v total 500µL per pellet) was added and metabolites were extracted in the aqueous phase (4). The protein content of the cell pellet was estimated by the Lowry method. The extracted sample was then lyophilized and prepared for MRS analysis in the same manner as for tumors described below.

Under the surgical plane of anesthesia (3% isoflurane in air 1L/min), the tumor was excised, freeze-clamped between a pair of aluminum tongs which were pre-cooled in liquid

nitrogen, and stored at -80°C until extraction. To extract soluble metabolites, the frozen tissue was first weighed (the weight ranges from 0.14-0.74 g) and then pulverized in liquid nitrogen using a suitable size mortar/pestle. Perchloric acid (PCA, 6.6%) was added at 3 mL per gram of tissue to the pulverized sample. The sample was then thawed and homogenized followed by centrifugation (11,000 g for 20 min at 4°C). The pH of the supernatant was adjusted to 7.0 ±0.2, using KOH and PCA in graded concentrations. The precipitated potassium perchlorate was removed by centrifugation (1000 ×g for 10 min at 4°C). The sample was then lyophilized and stored at -80°C freezer.

For high resolution ^{1}H MRS, the lyophilized powder was dissolved in 0.5 mL D_2O and filtered into a 5 mm (diameter) NMR tube. A glass capillary containing trimethylsilyl propanoic acid (TMSP) reference solution was calibrated inside the NMR tube to be 0.408 mM. ^{1}H MRS was performed on a 400 MHz vertical bore spectrometer (Avance DMX 400, Bruker Instruments Inc., Germany) using a 5-mm radio-frequency coil. ^{1}H MR spectra were analyzed by NUTS® software (Acorn NMR, Livermore, CA) with the reference peak set at 0 ppm. The integration of metabolite resonance peak(s) in an MR spectrum is proportional to its concentration. To determine the Glu concentration in the NMR tube, the integrated Glu-H4 resonance peaks (spread over 2.32-2.38 ppm) relative to the reference peak was multiplied by 0.408 mM. Multiplying the Glu concentration by sample volume (0.5 mL) gave the amount of Glu in the sample. The amount of Glu was normalized to the tissue wet weight or total protein in the cell pellet, yielding Glu concentration in the tumor tissue (µmole/gram) and cells (µmole/gram of protein), respectively. In spectra obtained from the 400 MHz MR spectrometer, there was no overlap of glutamine-H4 peaks with Glu-H4 peaks (5) or with other metabolites at the detection sensitivity of MRS, assuring no contamination in the estimation of Glu concentration.

Reference

1. Zhou R, Pantel AR, Li S, Lieberman BP, Ploessl K, Choi H, et al. [18F](2S,4R)4-Fluoroglutamine PET Detects Glutamine Pool Size Changes in Triple-Negative Breast Cancer in Response to Glutaminase Inhibition. Cancer Res 2017;77(6):1476-84.
2. Gross MI, Demo SD, Dennison JB, Chen L, Chernov-Rogan T, Goyal B, et al. Antitumor activity of the glutaminase inhibitor CB-839 in triple-negative breast cancer. Mol Cancer Ther 2014;13(4):890-901.
3. Nath K, Nelson DS, Heitjan DF, Leeper DB, Zhou R, Glickson JD. Lonidamine induces intracellular tumor acidification and ATP depletion in breast, prostate and ovarian cancer xenografts and potentiates response to doxorubicin. NMR Biomed 2015;28(3):281-90.
4. Le Belle JE, Harris NG, Williams SR, Bhakoo KK. A comparison of cell and tissue extraction techniques using high-resolution 1H-NMR spectroscopy. NMR Biomed 2002;15(1):37-44.
5. de Graaf RA. In Vivo NMR Spectroscopy – 2nd Edition: Principles and Techniques. John Wiley & Sons, Ltd; 2007.

Supplemental Table-1

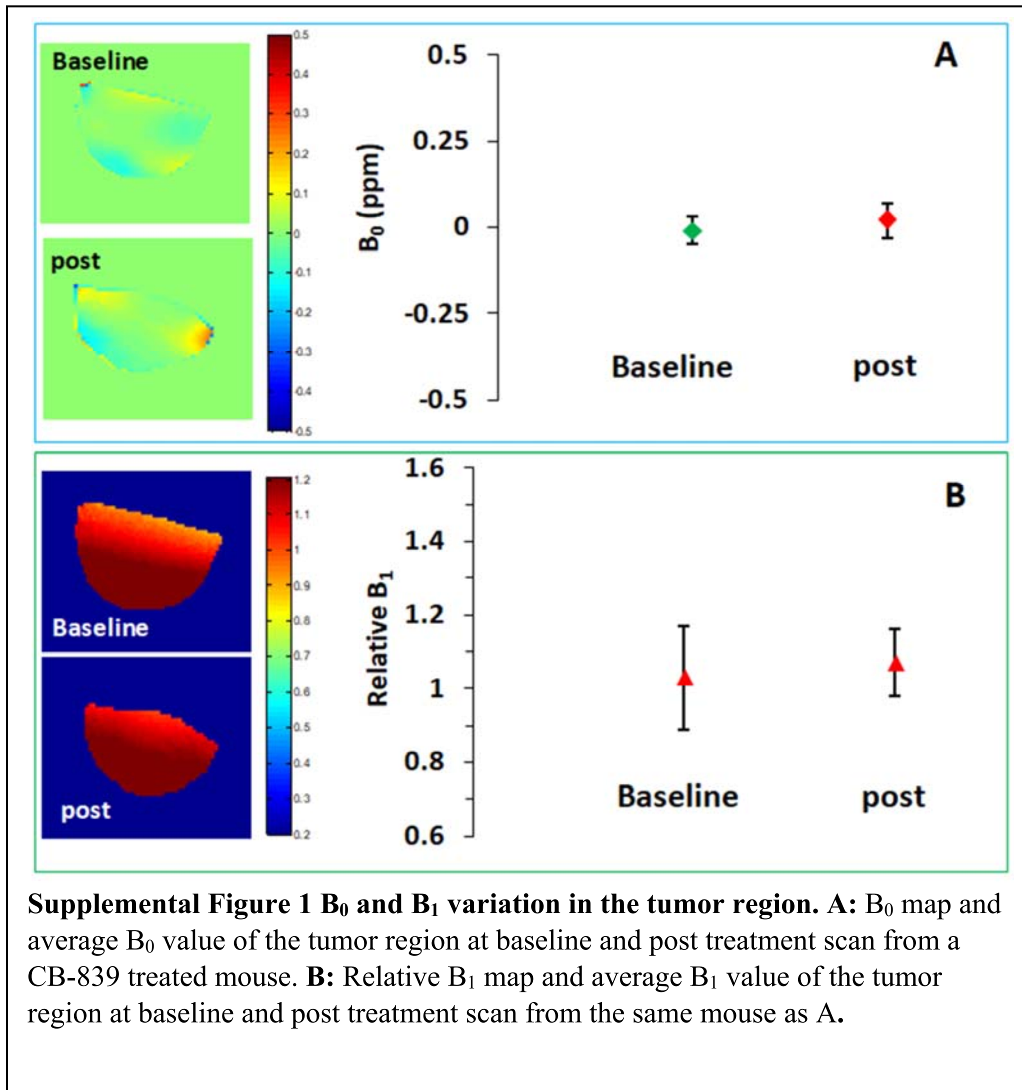
Changes of tumor metabolite concentration in CB839 and VEH treated mice

μmol/gwwt (mM)	**Glu**	Gln	Glu: Gln	Ala	lactate	tCr	tChol
VEH (n=10)	2.08 (± 0.25)	0.53 (± 0.10)	5.02 (± 0.97)	1.40 (± 0.21)	8.52 (± 0.80)	1.51 (± 0.15)	1.29 (± 0.14)
CB-839 (n=11)	0.68 (± 0.14)[a]	1.36 (± 0.23)[b]	0.61 (± 0.17)[b]	1.27 (± 0.09)[c]	8.67 (± 0.71)[c]	1.21 (± 0.07)[c]	1.08 (± 0.09)[c]

Data is presented as mean ± standard error of the mean (sem). gwwt: gram in wet weight (of tissue), Gln= glutamine, tCr = total creatine, tChol = total choline.

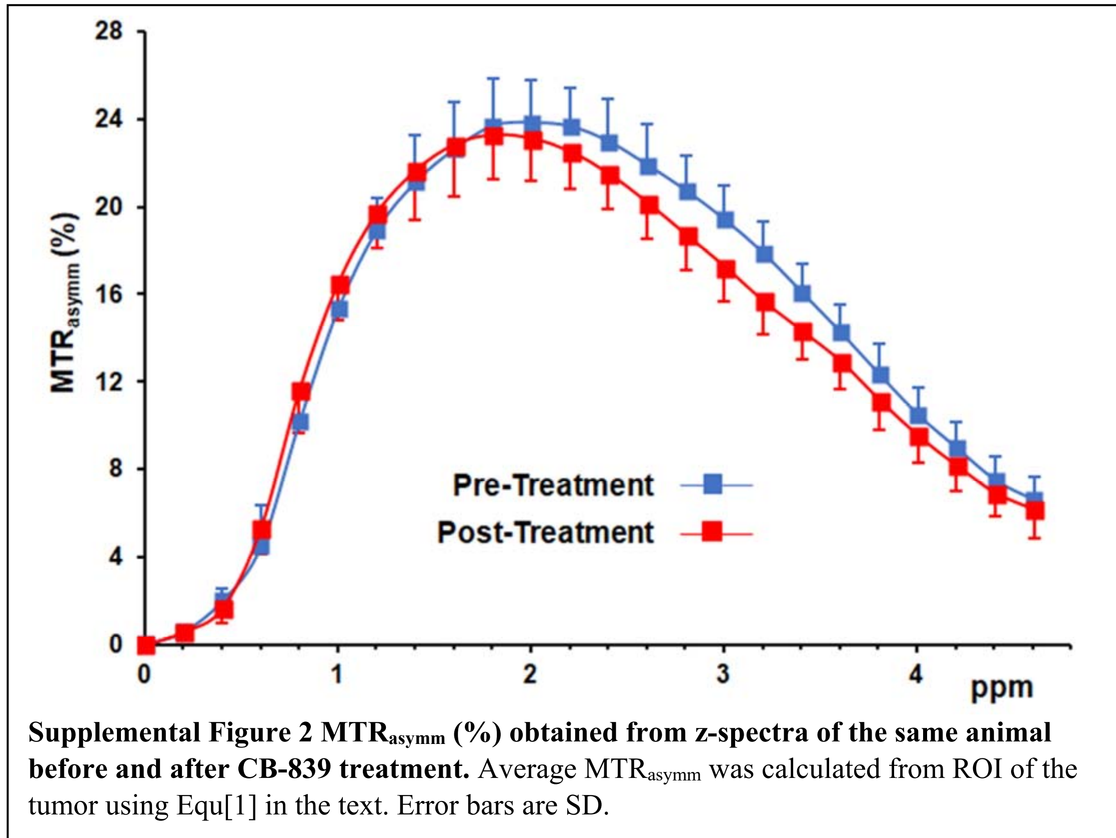
a: $P = 5.4 \times 10^{-5}$ compared to VEH group. b: $P < 0.01$. c: $P > 0.05$.

Supplemental Figure 1 **B_0 and B_1 variation of the tumor for GluCEST scan**



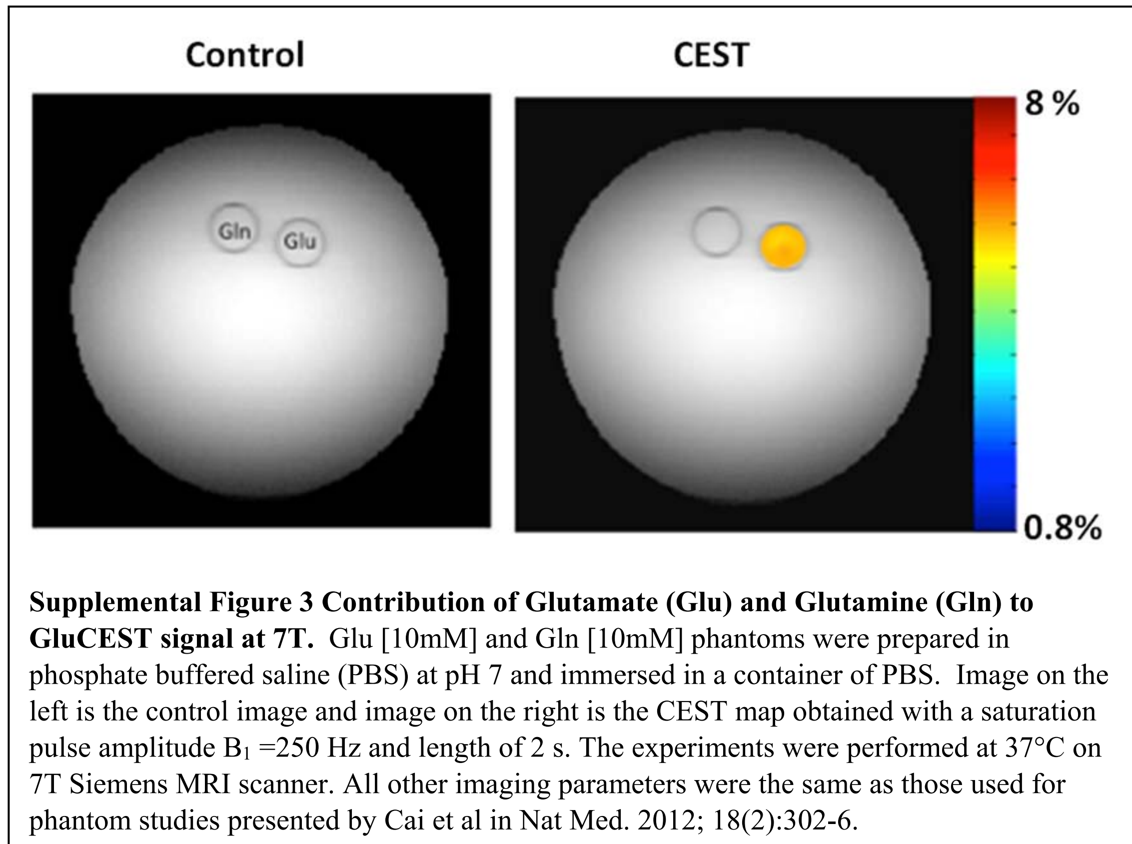
Supplemental Figure 1 B_0 and B_1 variation in the tumor region. A: B_0 map and average B_0 value of the tumor region at baseline and post treatment scan from a CB-839 treated mouse. **B:** Relative B_1 map and average B_1 value of the tumor region at baseline and post treatment scan from the same mouse as A**.**

Supplemental Figure 2 **MTR_{asymm} (%) obtained from z-spectra of the same animal before and after CB-839 treatment**



Supplemental Figure 2 MTR_{asymm} (%) obtained from z-spectra of the same animal before and after CB-839 treatment. Average MTR_{asymm} was calculated from ROI of the tumor using Equ[1] in the text. Error bars are SD.

<u>Supplemental Figure 3</u> Contribution of Glutamate (Glu) and Glutamine (Gln) to GluCEST signal at 7T.



Supplemental Figure 3 Contribution of Glutamate (Glu) and Glutamine (Gln) to GluCEST signal at 7T. Glu [10mM] and Gln [10mM] phantoms were prepared in phosphate buffered saline (PBS) at pH 7 and immersed in a container of PBS. Image on the left is the control image and image on the right is the CEST map obtained with a saturation pulse amplitude B_1 =250 Hz and length of 2 s. The experiments were performed at 37°C on 7T Siemens MRI scanner. All other imaging parameters were the same as those used for phantom studies presented by Cai et al in Nat Med. 2012; 18(2):302-6.